IMPROVING HEALTH AND PERFORMANCE IN HOLSTEIN DAIRY HEIFERS

A Dissertation

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by

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IMPROVING HEALTH AND PERFORMANCE IN HOLSTEIN DAIRY HEIFERS

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Given the limited knowledge surrounding calf-rearing systems a series of studies were developed to address some of the aspects regarding colostrum and whole milk (non-saleable or hospital milk) physical process (Chapter 1), supplementation of trace minerals (Chapter 2), non-antimicrobial alternatives to prevent and control neonatal calf diarrhea (Chapter 3 and 4), antimicrobial metaphylactic interventions in high-risk group of animals (Chapter 5), and pulmonary lesions in dairy heifers at weaning (Chapter 6).

Alternatives to heat-treatment of colostrum and non-saleable milk using ultraviolet light herein explored showed decreased levels of immunoglobulins in colostrum. Ultraviolet light and heat treatment decreased microorganism contamination. However, colostrum and non-saleable milk treatments were not associated with calf survivability, incidence of diseases (diarrhea and pneumonia), and average daily weight gain during the pre-weaning period. Injectable trace minerals supplementation improved leukocyte function and oxidative stress levels in dairy calves. However, these benefits did not translate to improved growth performance and health status for dairy calves in the first 60 days of life.

Enteric-coated crofelemer extract, a natural-product with anti-secretory properties produced an increase in fecal dry matter in diarrheic neonatal calves in a

challenge study. Furthermore, when the natural extract was administered prophylactically for the first two weeks of life, milk fed calves experienced fewer events of diarrhea and reduced use of fluid therapy when compared to non-treated calves. A metaphylactic intervention using a synthetic long acting macrolide decreased the combined incidence of respiratory disease and otitis in high-risk group-housed replacement heifers during the pre-weaning period. Finally, nulliparous heifers suffered consequences from lung consolidation detected at weaning through a decrease in reproductive performance and an increase in culling risk.

There are many important aspects on prevention and treatment of diseases affecting replacement dairy heifers. Strategically, different approaches can be used to improve replacement heifers' health and performance.

BIOGRAPHICAL SKETCH

Andre Gustavo Vieira Teixeira was born and raised in Goiania, Goiás - Brazil. He received his degree in Veterinary Medicine in 2009 from the School of Veterinary Medicine at the Federal University of Goiás, Brazil. In 2009, six months before his graduation, he was invited to join Dr. Rodrigo Bicalho's research group at Cornell University as a visiting scholar.

After the completion of his DVM training, he worked as a Research Assistant at Cornell University under the supervision of Dr. Bicalho. During that period, his training was through hands-on activities conducting applied research in large commercial dairy farms with emphasis on replacement dairy heifers.

In 2012, Andre Teixeira was accepted in the field of Animal Science at Cornell University to start his Ph.D. program, under the advisement of Dr. Rodrigo Bicalho. His PhD committee consisted of three additional members Dr. Daryl Nydam, Dr. Michael Van Amburgh, and Dr. Jessica A. A. McArt.

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I wouldn't be able to express my gratitude with words, in any language. This dissertation is not only a collective of studies, this represents a mutual effort of all my mentors, friends, and family that supported me during this journey.

My parents, Ary and Maria Augusta, my brother Luis Otávio, and my sisters Mayra, Arissa, and Luyara for all the love, respect, and encouragement they gave me to move abroad and pursue a dream. I could not have done it without them.

I have been extremely fortunate in my life to have friends who have showed me absolute support. Thanks to my long time roommates and friends Luciano Caixeta and Vinicius Machado for being there whenever I needed a friend. A special thanks to the Bicalho's family Rodrigo, Marcela, Felipe, and Rafael for allowing me into their lives. Nonetheless, I would like to also say thanks to my fellow graduate students who have made my daily work truly enjoyable.

To the members of my committee, Dr. Daryl Nydam, Dr. Jessica McArt, and Dr. Mike Van Amburgh for their patient support and guidance throughout my PhD program. I am extremely fortunate to have worked with three outstanding intellectuals.

To my advisor Dr. Rodrigo Bicalho, I am grateful for the trust, inspiration, and mentorship. Without his endless effort, knowledge, and wisdom I could not have completed this journey.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

LIST OF ABBREVIATIONS

ADG, average daily weight gain
AI, artificial insemination
ANOVA, analysis of variance
BHBA, β-hydroxybutyrate
BW, body weight
CI, confidence interval
CP, crude protein
CV, coefficient(s) of variation
DHI, Dairy Herd Improvement
DHIA, Dairy Herd Improvement Association
DIM, days in milk
DM, dry matter
DMI, dry matter intake
DNA, deoxyribonucleic acid
DNase, deoxyribonuclease
EDTA, ethylenediaminotetraacetate
ELISA, enzyme-linked immunosorbent assay
FDA, Food and Drug Administration
HTST, high temperature, short time
Ig, immunoglobulin
LPS, lipopolysaccharide
LSM, least squares means
mRNA, messenger ribonucleic acid
NDF, neutral detergent fiber
NRC, National Research Council
PCR, polymerase chain reaction
RNA, ribonucleic acid
RNase, ribonuclease
rRNA, ribosomal ribonucleic acid
SAS, Statistical Analysis System
SCC, somatic cell count
SD, standard deviation
SE, standard error
SEM, standard error of the mean
TMR, total mixed ration
TPC, total plate count
USDA, United States Department of Agriculture
UV, ultraviolet

CHAPTER 1

HEAT AND ULTRAVIOLET LIGHT TREATMENT OF COLOSTRUM AND HOSPITAL MILK: EFFECTS ON COLOSTRUM AND HOSPITAL MILK CHARACTERISTICS AND CALF HEALTH AND GROWTH PARAMETERS

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ABSTRACT

The aim of this study was to evaluate the effects of different physical treatments of bovine colostrum and hospital milk (non-saleable) on milk bacteriology, immunoglobulin G (IgG) and lactoferrin concentrations, calf serum IgG concentrations and calf health, growth, and survivability. Pooled colostrum samples (n = 297) were heat treated (HTC; 63 °C for 60 minutes), exposed to ultraviolet light (UVC; 45 J/cm^2) or untreated ('raw' colostrum, RC). Hospital milk (n = 712) was subjected to high temperature short time pasteurization (HTST; 72 °C for 15 seconds) and ultraviolet light irradiation (UVH; 45 J/cm^2). Neonatal Holstein heifer calves (n = 875) were randomly enrolled into one of three colostrum treatment groups; 309 in the HTC, 285 in the UVC, and 281 in the RC. Once enrolled into a colostrum treatment, heifers were block randomized (by colostrum treatment) into two hospital milk treatments HTST (RC = 144, HTC = 152, and UVC = 153) or UVH (RC = 137, HTC = 157, and UVC = 132).

Heat treatment of colostrum was more effective than UVC and HTST was more effective than UVH in reducing bacterial counts. Immunoglobulin-G and lactoferrin concentrations were significantly lower in HTC and UVC than in RC. Lactoferrin concentrations were significantly lower in HTST than in UVH or untreated hospital milk. There were no significant differences in serum IgG concentrations among calves fed HTC, UVC or RC. Colostrum and hospital milk treatments did not have any significant effect on calf body weight gain, survivability, or frequency of diarrhea or pneumonia.

INTRODUCTION

Colostrum provides calves with immunoglobulins (Igs), non-specific immune factors and nutrients (Weaver et al., 2000), but may also expose calves to pathogens (Stabel et al., 2004). Bacteria from contaminated colostrum may reduce the efficiency of IgG absorption (James et al., 1981; Johnson et al., 2007). High temperature short time (HTST) treatment of colostrum was associated with a decrease of 22-27% in IgG concentration regardless of the temperature regime (Stabel et al., 2004); however, when colostrum was heat treated at a lower temperature (60 °C) for 60 minutes, the IgG concentration did not change significantly when compared to raw colostrum (Johnson et al., 2007). A recent multi-herd study demonstrated similar results (Donahue et al., 2012). The viability of pathogens, such as *Mycobacterium avium* subsp. paratuberculosis (MAP), *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* serovar Enteritidis, was significantly reduced or eliminated in spiked colostrum after treatment at 60 °C for 60 minutes (Godden et al., 2006).

Feeding calves with milk replacer can be costly and the use of pasteurized, non-saleable milk (hospital milk) is an attractive alternative. Hospital milk includes milk from mastitic cows, which can have increased bacterial contamination. Pasteurization (63 °C for 30 minutes or 72 °C for 15 seconds) can reduce the counts of MAP in inoculated milk (Gao et al., 2002). On-farm pasteurization of milk effectively destroys MAP, Salmonella spp. and Mycoplasma spp. in spiked milk (Stabel et al., 2004).

Ultraviolet (UV) light disinfection systems are commonly used for water and waste water treatment in the USA and Europe (Lindenauer and Darby, 1994; Guo et

al., 2009). Ultraviolet light destroys several pathogens in drinking water (Hijnen et al., 2006) and *Staphylococcus aureus* in milk and *Listeria monocytogenes* in raw goat's milk (Matak et al., 2005; Krishnamurthy et al., 2007;), although UV irradiation of milk spiked with MAP did not result in an adequate reduction in infectivity (Donaghy et al., 2009).

The aims of this study were to determine the effect of heat and UV treatment on (1) bacterial counts and concentrations of lactoferrin and IgG in colostrum and hospital milk; (2) serum IgG concentrations in calves; and (3) calf survivability, pneumonia, diarrhea and body weight gain in the pre-weaning period.

MATERIALS AND METHODS

Farm and Management

The study was conducted from February until November 2011 at a commercial dairy farm that milked 2,800 Holstein cows near Ithaca, New York, USA. Immediately after parturition, calves were removed from maternity pens and placed in dry sawdust bedded pens. Neonatal calves were transported twice daily from the maternity area to the calf barn, which was a greenhouse-type building, with eight rows of 40 individual pens (each 1.7 meters long and 1.2 meters wide) isolated by plastic panels and bedded with a deep gravel base covered with pine shavings. Calves were kept in the same pen until weaning. Water and calf starter were available ad libitum from day one until calves were moved from the calf barn to a post-weaning heifer pen. Calves were weaned at approximately 45 days of age by a progressive reduction in milk over 5 days.

Study design and data collection

On the basis of a priori sample size calculations, it was estimated that, with a sample size of 270 calves per colostrum treatment group, an average daily weight gain (ADG) of 660 g (with a standard deviation of 131 g) would permit detection of differences in daily body weight gain $\geq$ 32 g. In addition, it was estimated that a sample size of 270 calves per colostrum treatment would permit detection of a difference in frequency of diarrhea of 12% between treatment groups, considering a baseline frequency of diarrhea of 50%. Sample size calculations were based on an α value of 0.05, confidence of 0.80 and a two-tailed t test.

A randomized field trial study design was used. All heifers born from February to October 2011 were eligible for enrolment in the study. All calves received 4 L colostrum within 4 hours of birth by esophageal tubing (Oral Calf Feeder Bag with Probe, Jorvet). Colostrum treatments were pre-assigned by a random table generated in Excel (Microsoft) using the random number function. To prepare equal aliquots of pooled colostrum for each of the three treatments, a minimum volume of 45 L was needed. Colostrum was harvested twice daily and stored in a refrigerator (1.7 °C) until the desired volume was reached, which was achieved within 36 hours. The pooled colostrum (45 L or greater) was firstly homogenized and then divided into three equal aliquots of at least 15 L each; one third was untreated; one third was heat treated at 63 °C for 60 minutes using a batch pasteurizer (DT-10G Platinum, Dairy Tech) and the last third was treated with UV light (UV Pure system 45 J/cm^2, GEA Farm Technologies). All treated colostrum was stored in 4 L containers and refrigerated (1.7 °C) until needed.

Calves were further blocked by colostrum treatment and randomized into one of two hospital milk treatments: HTST (72 °C for 15 seconds) or UVH (45 J/cm^2). Hospital milk was harvested twice daily and stored in a stainless steel milk tank (600 L) mounted on a transport truck and refrigerated at 5 °C until it was transported to the pasteurization room (located at the calf barn), where it was divided into aliquots and treated according to the study protocol. Calves assigned to the HTST group received 6 L/day pasteurized hospital milk (Terminator, Goodnature Products). Calves allocated to the UVH group were fed 6 L/day hospital milk treated with UV light (UV Pure system, GEA Farm Technologies).

All machinery was sanitized prior to and after each running cycle. Both the UV-Pure system and the HTST pasteurizer are pre-set with their own clean-in-place procedure. The cleaning procedure comprises a pre-rinse cycle with warm water, a high pressure washing cycle with hot water and an alkaline detergent (TRI-PFAN, GEA Farm Technologies), and a final rinse with warm water and a low foam acid cleaner (LAC, GEA Farm Technologies). The colostrum containers (plastic jars) and the batch pasteurizer (DT-10G Platinum, Dairy Tech) were cleaned with an initial rinse with warm water, followed by a hand-brush wash using alkaline detergent, then a final rinse with warm water and a low-foam acid cleaner.

A blood sample was collected from each calf at 3 days of age; serum was harvested after centrifugation at 2,500 g for 10 minutes and stored at -80 °C. Birth weight, weekly measures of fecal scores and body weight were recorded for each calf until 60 days of age.

Case definitions

All health-related events during the study period were recorded. Retained placenta was defined as a condition where cows failed to release their fetal membranes within 24 hours of calving. Metritis was defined as the presence of fetid, watery, red-brown uterine discharge. Pneumonia was defined when two or more of the following clinical signs were detected in a calf: cough, rectal temperature > 39.5 °C, respiratory rate > 40 breaths/minutes, increased cranioventral lung sounds or wheezes. Calves were recorded as diarrheic when the fecal consistency was watery and fetid.

Microbiological assays

Hospital milk samples were collected before and after UVH treatment and HTST pasteurization twice daily for 5 days per week (January to November 2011). Colostrum samples were collected pre-processing (after pooling) and post-processing for each day (January to September 2011). Samples (50 mL) were collected, placed on ice and transported to the laboratory to be processed daily. Samples of colostrum and milk were homogenized, diluted serially (10^{-1} to 10^{-12}) and 20 μL aliquots were plated on standard aerobic medium (EMD Millipore) for total bacterial counts (TBCs), and on specific chromogenic media for detection and enumeration of *Escherichia coli*, *Staphylococcus aureus*, and group B Streptococcus spp. (CHROMagar). Plates were incubated aerobically for 24 hours at 37 °C. All cultures were performed in triplicate. The number of colony forming units (CFUs)/mL was made by calculating the average number of colonies (from triplicates) and multiplying this number by the appropriate dilution factor.

ELISA assay

To quantify immunoglobulin-G in the serum of calves and colostrum a commercial ELISA kit was used (Immuno-Tek Bovine IgG) and to determine lactoferrin in colostrum and hospital milk another commercial ELISA kit was used (Bethyl Laboratories). All colostrum and hospital milk samples were thawed and homogenized, aliquots of 1.5 mL were centrifuged at 10,000 g for 15 minutes at 4 °C, and the supernatant was collected and used to perform the aforementioned assays.

Statistical analysis

Descriptive statistics and univariable analysis were performed using SAS. Analysis of variance (ANOVA) was used to evaluate the effect of hospital milk and colostrum treatments on the log reduction of CFU and the effect of colostrum treatment (RC, HTC or UVC) on IgG and lactoferrin concentrations using the MIXED procedure in SAS.

To analyze the effect of colostrum treatment on calf serum IgG concentrations at 3 days of life, a general linear model was fitted to the data using SAS. The outcome variable was calf serum (g/L), which was modelled as a Gaussian (normally distributed data) variable. The model assumption that the residuals were normally distributed was assessed and satisfied by visual evaluation of the distribution plot of the Studentized residuals. Other independent variables offered to the model were parity of the dam (primiparous or multiparous), location of parturition (maternity pen or pre-fresh free stall - where dry cows were housed during 3 week before expected calving date), calving ease of the dam (assisted or non-assisted), gestation length of

the dam (days), birth weight quartile, and dam postpartum health events (metritis, displaced abomasum and retained placenta).

The only calf-related risk factors offered to the models were those observed before enrolment (e.g. post-treatment colostrum bacterial CFU was not offered). However, post-parturition dam-related risk factors, such as postpartum diseases, were included in the regression models. All possible two-way interaction terms between treatments and all independent variables were evaluated in the model. Manual backward variable elimination considering main effects and two-way interactions of colostrum treatment with all the other independent variables was performed. Variables and interaction terms were retained in the model when $P \leq 0.05$.

The effect of treatment on calf survival was analyzed by Cox's proportional hazard model in SAS. Calves were right-censored if they were alive at the end of the data collection period (60 days). Variables offered to the models included colostrum treatment (RC, UVC or HTC), hospital milk treatment group (UVH or HTST), parity of the dam (primiparous or multiparous), calving location (maternity pen or pre-fresh free stall), calving ease of the dam (assisted or non-assisted), gestation length (days), dam post-partum health events (metritis, displaced abomasum and retained placenta) and calf birth weight quartiles (first: 22 to 37 kg; second: 38 to 41 kg; third: 39 to 44 kg; fourth: 45 to 59 kg). All possible two-way interaction terms between treatments and all independent variables were evaluated in the model, as well as the two-way interaction between colostrum and waste milk treatment. Backward variable elimination (Cantor, 1997) was undertaken considering main effects and two-way interactions of colostrum and waste treatment with all the other independent variables.

Variables and interaction terms were retained in the model when $P \leq 0.05$.

The effects of colostrum and hospital milk treatments on diarrhea and pneumonia were evaluated by logistic regression models that were fitted in Stata (StataCorp LP); the dependent variables for these models were occurrence of diarrhea or pneumonia (no = 0 or yes = 1) over the entire observation period (60 days). The independent variables offered to this model were the same as described above for the other multivariable models. Interaction terms and variable selection methodology were evaluated as described for the Cox's Proportional Hazard model. Adjusted probabilities were calculated from the model for all categorical variables retained in the model using the adjusted probability option in Stata.

A mixed general linear model was fitted to the data using SAS software to analyze the effect of colostrum and hospital milk treatment groups on repeated measures of calf ADG per week. Calf daily weight gain was calculated for each week by subtracting the latest body weight from the previous week's body weight and dividing it by seven; therefore, the outcome variable was a series ADG, which was modeled as a Gaussian (normally distributed data) variable. The assumption that the residuals were normally distributed was assessed by visually evaluating the distribution plot of the Studentized residuals. The independent variables offered to the model were colostrum treatment group (RC, UVC or HTC), hospital milk treatment group (UVH or HTST), week since birth (0 to 8 weeks), parity of the dam (primiparous or multiparous), gestation length of the dam (days), calving location (maternity pen or pre-fresh free stall) and dam post-partum health events (metritis, displaced abomasum, and retained placenta). The only calf related risk factors offered

to the models were those observed before enrolment (e.g. post-treatment colostrum bacterial CFU was not offered). However, post-parturition dam-related risk factors, such as postpartum diseases, were included.

The calf daily weight gains were collected longitudinally and consisted of a total of eight measurements per calf; the first calf weight measurement was collected within 12 hours of birth and subsequently eight other weekly measurements were collected until weaning. Since weight measurements were repeated measures within each calf, the error term was modeled by imposing a first order autoregressive covariance structure to appropriately account for within-calf correlation. All possible two-way interaction terms between treatments and all independent variables were evaluated in the model, as well as the two-way interaction between colostrum and waste milk treatment. Backward variable elimination considering main effects and two-way interactions of colostrum and waste treatment with all the other independent variables was performed. Variables and interaction terms were retained in the model when $P \leq 0.05$. A separate model, similar to the model described above, was used to evaluate the effect of calf diseases on weekly weight gain. For all general linear models and mixed general linear models, the least square means (LSM) are reported.

RESULTS

Descriptive statistics

Descriptive statistics for calf- and dam-related events are presented in **Table 1.1**.

Bacteriology, IgG and lactoferrin concentrations in colostrum and hospital milk

Log reductions in CFUs were significantly lower in HTC than UVC for TBCs, *Escherichia coli*, and Streptococcus spp. There was no difference between HTC and UVC in the CFU log reduction values for S. aureus. Colony forming units for total bacteria count were significantly lower in HTST than UVH ($P < 0.01$). There was no significant difference between UVH and HTST in CFU log reduction values for *Escherichia coli* and Streptococcus spp. ($P = 0.88$; **Table 1.2**). For *Staphylococcus aureus*, cultures only identify *S. aureus* during the months of March and April.

There were significantly lower IgG concentrations in HTC (52.4 g/L; 95% confidence interval, CI, 48-57 g/L) and UVC (39.5 g/L; 95% CI 35-44 g/L) compared to RC (69.1 g/L; 95% CI 64-74 g/L; $P < 0.001$; **Figure 1.1**).

Table 1.1: Descriptive statistics for calf and dam related events by treatment groups. Ultraviolet light treatment of colostrum (UVC), heat treatment of colostrum (HTC), ultraviolet light treatment of hospital milk (UVH) and high temperature, short time pasteurization of hospital milk (HTST).

		RC		UVC		HTC	
		UVH	HTST	UVH	HTST	UVH	HTST
n		137	144	132	153	157	152
Calves born to multiparous cows, %		57%	56%	50%	58%	50%	55%
Calves born to assisted parturition, %		6%	12%	10%	13%	8%	8%
Calves born in the free-stall, %		18%	17%	20%	18%	15%	17%
Calf diarrhea, %		41%	43%	34%	42%	36%	34%
Calf pneumonia, %		14%	11%	10%	8%	8%	9%
Calf mortality, %		6%	3%	4%	4%	4%	5%
Calf morbidity, %		92%	94%	93%	95%	96%	93%
Dam diagnosed with retained placenta, %		4%	6%	4%	4%	6%	3%
Dam diagnosed with metritis, %		16%	10%	11%	12%	17%	9%
Dam diagnosed with displaced abomasum, %		2%	1%	2%	1%	3%	2%
Calves' birth weight, kg		41.6 (5.2)	42.4 (5.7)	42.5 (5.8)	42.2 (5.5)	41.5 (5.7)	41.0 (5.0)
Calves' serum immunoglobulin-G, g/L		22.2 (1.3)	23.0 (1.7)	20.2 (1.2)	20.2 (1.3)	23.0 (1.3)	23.3 (1.2)
Gestation length, days		276 (6.1)	276 (6.3)	276 (6.3)	276 (7.0)	276 (4.9)	277 (4.2)
Average daily weight gain, g/day		677 (53)	683 (55)	685 (63)	669 (61)	664 (53)	690 (53)
Pre-treatment milk, CFU/mL	TBC	17×10^6	14×10^6	17×10^6	16×10^6	15×10^6	15×10^6
	Escherichia coli	11×10^6	9.4×10^6	9.9×10^6	9.3×10^6	7.6×10^6	15×10^6
	Streptococcus spp.	7.8×10^5	9.1×10^5	7.9×10^5	8.2×10^5	8.8×10^5	10×10^6

TBC, Total bacteria count for enumeration of total aerobic bacteria using standard medium
Escherichia coli, Detection and enumeration of *Escherichia coli* using chromogenic medium
Streptococcus spp., Detection and enumeration of group B Streptococcus spp. using chromogenic medium

Table 1.2: Average log reductions in bacterial colony counts for ultraviolet light treatment of colostrum (UVC), heat treatment of colostrum (HTC), ultraviolet light treatment of hospital milk (UVH) and high temperature, short time pasteurization of hospital milk (HTST).

	Mean (SD)			
	Colostrum (n = 297)		Hospital Milk (n=712)	
	UVC	HTC	UVH	HTST
TBC	1.7 (1.0) [a]	3.5 (1.5) [b]	3.3 (1.8) [a]	5.2 (1.1) [b]
Escherichia coli	2.2 (1.9) [a]	4.5 (2.7) [b]	1.7 (1.7) [a]	1.2 (1.8) [a]
Staphylococcus aureus	0.4 (1.2) [a]	0.4 (1.2) [a]	0.2 (0.8) [a]	0.2 (0.8) [a]
Streptococcus spp.	2.5 (1.9) [a]	2.5 (1.9) [a]	2.0 (2.2) [a]	2.4 (2.3) [a]

[a,b], Different letters within the same column indicate statistically significant differences ($P<0.05$).
UVC, Ultraviolet light colostrum treatment
HTC, Heat treatment of colostrum (63 °C for 60 minutes)
UVH, Ultraviolet light hospital milk treatment
HTST, High temperature short time pasteurization of hospital milk (72 °C for 15 seconds)
Mean (SD), Mean post-treatment colony forming unit log reduction and standard deviation
TBC, Total bacteria count for enumeration of total aerobic bacteria using standard medium
Escherichia coli, Detection and enumeration of *Escherichia coli* using chromogenic medium
Staphylococcus aureus, Detection and enumeration of *Staphylococcus aureus* using chromogenic medium
Streptococcus spp., Detection and enumeration of group B Streptococcus spp. using chromogenic medium

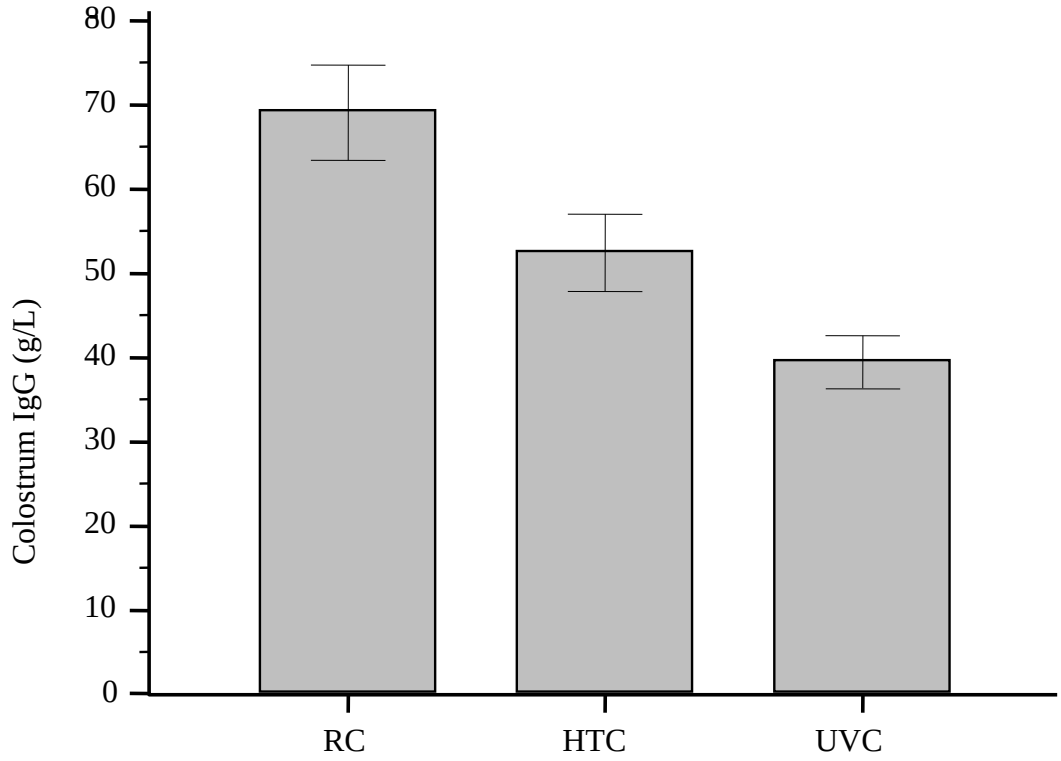

Figure 1.1: Average colostrum IgG and respective 95% confidence intervals by the different treatment groups. Raw colostrum (pre-process; RC), ultraviolet light treatment of colostrum (UVC), and heat treatment of colostrum (63 °C for 60 minutes; HTC). The average colostrum Immunoglobulin-G was 69.1 mg/mL (95% CI, 64-74 g/L), 52.4 mg/mL (48 – 57), and 39.5 mg/mL (35 – 44) for RC (n= 91), HTC (n = 94), and UVC (n = 96) respectively ($P < 0.001$). (52.4 g/L; 95% confidence interval, CI, 48-57 g/L) and UVC (39.5 g/L; 95% CI 35-44 g/L) compared to RC (69.1 g/L;; P < 0.001

Concentrations of lactoferrin were significantly lower in HTC and UVC than RC ($P < 0.001$) and in HTST compared to untreated hospital milk ($P < 0.01$), whereas there was no significant difference between UVH and untreated hospital milk ($P = 0.22$; **Figure 1.2**).

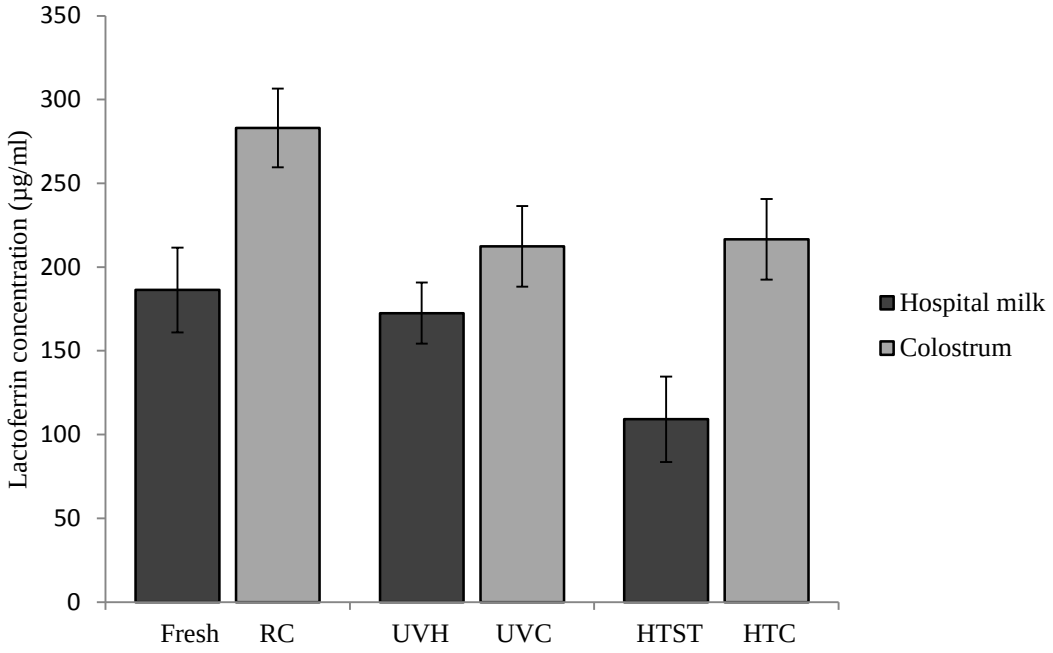

Figure 1.2: Average lactoferrin concentration and respective 95% confidence intervals by the different treatment groups. Raw colostrum (RC), ultraviolet light treated colostrum (UVC), and heat treated colostrum (63 °C for 60 minutes; HTC). Lactoferrin was also measured in fresh hospital milk (Fresh), ultraviolet treated hospital milk (UVH), and pasteurized hospital milk (HTST).

Serum IgG, disease, survivability and average daily gain of calves

There were no significant differences in serum IgG concentrations at 3 days of life between calves fed HTC, UVC or RC (P = 0.1; **Table 1.3**).

Table 1.3: Least squares means (LSMs) of calf serum immunoglobulin G (g/L) by categorical variables included in the analysis of variance (ANOVA).

		Regression Coefficient (± SE)	LSM (95% CI) of serum IgG (g/L)	*P*-value
Intercept		21.98 (1.26)		
Treatment	RC	Reference	22.5 (20.7-24.4)	0.1
	HTC	0.58 (1.28)	23.1 (21.4-24.8)	
	UVC	-2.02 (1.31)	20.5 (18.7-22.3)	
Birth weight quartiles, kg	22 to 37	-2.23 (1.44)	19.3 (17.3-21.3)	0.006
	38 to 41	1.99 (1.48)	23.5 (21.4-25.6)	
	42 to 44	2.46 (1.49)	23.9 (21.9-26.1)	
	45 to 59	Reference	21.5 (19.5-23.5)	

RC, Raw colostrum
HTC, Heat treatment of colostrum (63 ℃ for 60 minutes)
UVC, Ultraviolet-light treatment of colostrum

The variables colostrum and hospital milk treatments were not significantly associated with the odds of diarrhea or pneumonia. Parity of the dam, parturition assistance and birth weight quartile were significantly associated with the odds of diarrhea; calves in the smallest birth weight quartile had the highest odds of diarrhea, with an adjusted diarrhea frequency of 46% compared to an adjusted frequency of 35.7% for the calves in the highest birth weight quartile (P = 0.01; **Table 1.4**).

Table 1.4: Effect of colostrum and hospital milk treatments on the odds of diarrhea analyzed by multivariable logistic regression.

		Regression Coefficient (± SE)	Adjusted Probability %	Adjusted Odds Ratio (95% CI)	*P*-value
Intercept		-0.31 (0.12)			
Colostrum	RC	Reference	42.8	Reference	0.17
	HTC	-0.14 (0.1)	36.7	0.7 (0.5-1.0)	
	UVC	-0.03 (0.1)	40.2	0.8 (0.6-1.1)	
Hospital milk	HTST	Reference	38.6	Reference	0.82
	UVH	-0.02 (0.07)	37.1	1.0 (0.7-1.3)	
Calving	Natural	Reference	37.0	Reference	0.05
	Assisted	0.23 (0.12)	46.3	1.6 (1.0-2.5)	
Dam's Parity	Primiparous	Reference	32.1	Reference	0.001
	Multiparous	0.24 (0.07)	43.2	1.6 (1.2-2.2)	
Birth weight quartiles, kg	22-37	0.28 (0.12)	46.0	1.8 (1.2-2.4)	0.01
	38-41	0.15 (0.12)	44.5	1.6 (1.1-2.4)	
	42-44	-0.1 (0.13)	40.1	1.3 (0.8-1.9)	
	45-59	Reference	35.7	Reference	

RC, Raw colostrum
HTC, Heat treatment of colostrum (63 ℃ for 60 minutes)
UVC, Ultraviolet-light treatment of colostrum
HTST, High temperature short time pasteurization of hospital milk (72 ℃ for 15 seconds)
UVH, Ultraviolet-light treatment of hospital milk

Calves born to cows that developed metritis had 1.9 times higher likelihood of having pneumonia compared to calves born from cows that did not develop metritis (P = 0.04; **Table 1.5**).

Table 1.5: Effect of colostrum and hospital milk treatments on the odds of pneumonia analyzed by multivariable logistic regression.

		Regression Coefficient (± SE)	Adjusted Probability %	Adjusted Odds Ratio (95% CI)	*P*-value
Intercept		-1.43 (0.18)			
Colostrum	RC	Reference	12.3	Reference	0.37
	HTC	-0.16 (0.17)	8.6	0.68 (0.4-1.3)	
	UVC	-0.05 (0.17)	9.0	0.76 (0.4-1.3)	
Hospital milk	HTST	Reference	9.2	Reference	0.54
	UVH	0.07 (0.12)	10.7	1.1 (0.7-1.8)	
Metritis	No	Reference	8.4	Reference	0.04
	Yes	0.32 (0.16)	20.9	1.9 (1.0-3.5)	
Retained Placenta	No	Reference	8.5	Reference	0.001
	Yes	0.73 (0.19)	36.4	4.3 (2.1-9.0)	
Dam's parity	Primiparous	Reference	7.2	Reference	0.07
	Multiparous	0.22 (0.12)	12.3	1.6 (1.0-1.8)	

RC, Raw colostrum
HTC, Heat treatment of colostrum (63 ℃ for 60 minutes)
UVC, Ultraviolet-light treatment of colostrum
HTST, High temperature short time pasteurization of hospital milk (72 ℃ for 15 seconds)
UVH, Ultraviolet-light treatment of hospital milk

The variables colostrum and hospital milk treatment were not significantly associated with survivability. The independent variables parity of the dam, metritis of the dam and birth weight 'quartiles' were significantly associated with survivability (**Table 1.6**).

Table 1.6: Cox's proportional hazards survival analysis evaluating the effect of different risk factors on calf survival during the first 60 days of life (from birth until 60 days).

		Regression coefficient (±SE)	Mortality %	Hazard Ratio (95% CI)	*P*-value
Colostrum	RC	Reference	3.2	Reference	0.890
	HTC	0.09 (0.45)	3.8	1.1 (0.4-2.6)	
	UVC	-0.14 (0.49)	2.8	0.87 (0.3-2.3)	
Hospital milk	HTST	Reference	2.4	Reference	0.178
	UVH	0.53 (0.39)	4.2	1.7 (0.8-3.6)	
Dam's parity	Primiparous	Reference	1.9	Reference	0.015
	Multiparous	1.04 (0.43)	4.4	2.8 (1.2-6.5)	
Metritis	No	Reference	2.4	Reference	0.004
	Yes	1.14 (0.41)	9.5	3.2 (1.4-7.2)	
Birth weight quartiles, kg	22-37	1.23 (0.49)	6.5	3.4 (1.3-9.0)	0.008
	38-41	-0.73 (0.82)	1.4	0.5 (0.1-2.4)	
	42-44	0.30 (0.61)	2.6	1.3 (0.4-4.4)	
	45-59	Reference	2.3	Reference	

RC, Raw colostrum
HTC, Heat treatment of colostrum (63 ℃ for 60 minutes)
UVC, Ultraviolet-light treatment of colostrum
HTST, High temperature short time pasteurization of hospital milk (72 ℃ for 15 seconds)
UVH, Ultraviolet-light treatment of hospital milk

The variables colostrum and hospital milk treatment were not significantly associated with ADG. There was a significant association between pneumonia and ADG (**Table 1.7**).

Table 1.7: Effect of colostrum and hospital milk treatments, birth weight, pneumonia and calving location on average daily weight gain (ADG)**.** Average daily weight gain (g) was calculated for each week. Weight was measured weekly from birth until weaning for a total of 8 weeks.

		Regression coefficient (±SE)	Adjusted ADG (95% CI)	*P*-value
Intercept		795.5 (55.1)		
Birth weight		-3.25 (1.2)		0.01
Colostrum	RC	Reference	617.7 (582.8-652.6)	0.92
	HTC	-6.3 (16.1)	611.4 (577.2-645.7)	
	UVC	-4.2 (16.3)	613.5 (578.4-648.6)	
Hospital milk	HTST	Reference	613.9 (581.5-646.4)	0.96
	UVH	0.58 (13.1)	614.5 (582.5-646.5)	
Pneumonia	No	Reference	676.0 (655.3-696.7)	0.0001
	Yes	-123.6 (25.0)	552.4 (502.2-602.7)	
Calving location	Stall	Reference	594.3 (550.0-638.7)	0.06
	Maternity	39.8 (20.8)	634.1 (609.3-659.0)	

RC, Raw colostrum
HTC, Heat treatment of colostrum (63 ℃ for 60 minutes)
UVC, Ultraviolet-light treatment of colostrum
HTST, High temperature short time pasteurization of hospital milk (72 ℃ for 15 seconds)
UVH, Ultraviolet-light treatment of hospital milk

DISCUSSION

In this study, UV and heat treatments reduced CFU counts in colostrum and hospital milk. Heat treatment was generally more effective than UV treatment in decreasing bacteria counts in colostrum and hospital milk. HTC had lower TBCs and CFU counts for E. coli and Streptococcus spp., but not for S. aureus, than UVC. HTST had lower TBCs than UVH treatment, but there was no difference in CFU counts for E. coli and Streptococcus spp. between HTST and UVH.

Heat treatment of colostrum and HTST of hospital milk have been evaluated extensively as means to reduce bacterial contamination of colostrum and milk and, consequently, disease incidence in calves (Meylan et al., 1996; Grant et al., 2002; Stabel et al., 2004; Elizondo-Salazar et al., 2010; Donahue et al., 2012; Godden et al., 2012; Pearce et al., 2012). Our results are largely in agreement with the published literature and illustrate the high efficiency of HTC and HTST in reducing TBC and CFU counts for E. coli, S. aureus, and Streptococcus spp. UV light treatment of goats' milk reduces L. monocytogenes counts (Matak et al., 2005). No studies have been published that evaluate UV treatment of waste milk, specifically colostrum.

The composition and consistency of a liquid can affect UV light penetration and its bactericidal activity (Foley and Otterby, 1978; Lindenauer and Darby, 1994). The presence of dissolved and suspended solids can scatter UV light and provide a site for bacterial aggregation, attenuating the bactericidal activity of this form of radiation (Ye et al., 2007; Koutchma et al., 2004). Colostrum has a thicker consistency than hospital milk and higher percentages of total solids (23.9 vs. 12.9%), fat (6.7 vs. 4.0%) and protein (14.0 vs. 3.1%; Godden, 2008). Therefore, UV light penetration and the

rate of bacterial inactivation would be expected to be lower in colostrum than hospital milk. In our study, a direct comparison between UVC and UVH was not possible, since colostrum and hospital milk samples had different initial bacterial counts. Nevertheless, when evaluating the log reductions of bacterial counts in UVC vs. HTC and UVH vs. HTST, UV light appeared to be more effective in reducing bacterial counts in hospital milk than in colostrum.

HTC and UVC significantly decreased colostrum IgG concentrations compared to untreated colostrum, with mean IgG reductions of 24.2% and 42.8%, respectively. This finding is consistent with reported losses of 23.6-58.8% for a standard LTLT pasteurization protocol (63 °C for 30 minutes) (Godden et al., 2003). A reduction of 12.3% in colostrum IgG concentration was reported using a laboratory simulation of the LTLT method (63 °C for 30 minutes) (Meylan et al., 1996). Using a lower temperature (60 °C) for a longer period of time (60 minutes), there was no significant reduction in IgG concentrations in heat treated colostrum compared to raw colostrum (Donahue et al., 2012). Interestingly, ultraviolet light treatment of colostrum resulted in significantly lower IgG concentrations than in raw or heat-treated colostrum. The authors could not identify in the literature a possible justification for this reduction and further work to elucidate the mechanism for this effect is warranted.

In the present study, there were no significant differences in serum IgG concentrations at 3 days of life among calves fed HTC, RC or UVC. In an earlier study by Godden et al. (2003), calves fed 4 L of LTLT-treated colostrum (63 °C for 30 minutes) had no differences in serum IgG concentrations in comparison with calves

fed untreated colostrum. In a study by Johnson et al. (2007), colostrum treated at 60 °C for 60 minutes had reduced concentrations of bacteria compared to untreated colostrum, without a reduction in IgG concentrations; furthermore, calves fed heat treated colostrum had significantly higher serum IgG and total protein concentrations than calves fed raw colostrum.

James et al. (1981) reported a reduced uptake of γ-globulin when segments of calf intestine were exposed to bacteria when compared to calves receiving sterile inoculum. In our study, UV light treatment was less effective than heat treatment in reducing TBC and also resulted in a greater reduction in colostrum IgG concentration; however there was no significant difference in serum IgG concentrations in calves fed UV-treated, heat treated or untreated colostrum.

Lactoferrin binds and sequesters iron, acting as a bacteriostatic protein (Miyauchi et al., 1998; Baker and Baker, 2005). In a study by Joslin et al. (2002), calves fed milk replacer supplemented with lactoferrin (1 g/day) during the pre-weaning period had significant higher ADG than non-supplemented calves. In the present study, lactoferrin concentrations were decreased in heat and UV treated colostrum compared to RC. Pasteurization of hospital milk (HTST) is known to reduce concentrations of lactoferrin (Schwarcz et al., 2008). In this study HTST was associated with a significant reduction in lactoferrin concentrations. However, UVH did not significantly reduce lactoferrin concentrations in hospital milk.

Colostrum and hospital milk treatments did not affect the incidence of diarrhea. However, the parity of the dam, assisted parturition, and calf birth weight were significantly associated with calf diarrhea during the pre-weaning period. There

was a negative correlation between calves born from multiparous cows and the frequency of diarrhea. In contrast, an observational study by Perez et al. (1990), in which calves were fed their own mothers' colostrum, calves born from primiparous cows had an increased risk of developing diarrhea. In the present study, calves born from assisted parturitions were more susceptible to diarrhea during the pre-weaning period. In contrast, Sivula et al. (1996) and Lombard et al. (2007) reported a negative effect of assisted parturitions on the incidence of calf diarrhea in earlier life when compared to calves born from normal calving.

In this study, colostrum and hospital milk treatments did not affect the incidence of pneumonia. However, calves born from cows that subsequently developed metritis or retained placenta were more likely to have pneumonia up to 60 days of life. Parity of the dam was not associated with incidence of pneumonia. However, Perez et al. (1990) reported that calves born from primiparous cows were less prone to pneumonia. In a study by Lombard et al. (2007), calves born to primiparous cows had a reduced likelihood of a respiratory event compared to calves born to multiparous cows. Calves born to cows that had a disease 280-50 days before calving or cows with retained placenta at parturition were at a higher risk of developing pneumonia than were calves born to healthy cows (Lundborg et al., 2003).

The ADG of calves with pneumonia was 124 g/day less than unaffected calves and they were on average 5 kg lighter at weaning. Similarly, Virtala et al. (1996) reported that calves treated for pneumonia had a reduction of 66 g/day in ADG. Lundborg et al. (2003) also reported a negative effect of respiratory disease on calf growth.

Survivability of calves was affected by parity of the dam, birth weight of the calf and metritis of the dam, but was not affected by colostrum or hospital milk treatment. Calves with a birth weight of 38-41 kg were less likely to die before weaning. Similarly, Henderson et al. (2011), observed that an 'arrival weight' (weight recorded within 2 days of birth for calves entering rearing facilities) of 38-41 kg was optimal for calf survivability. In the present study, calves born from cows that developed metritis were more susceptible to pneumonia and this could be the reason why metritis of the dam was also associated with calf survivability.

There were no significant differences on calf health and growth between colostrum and hospital milk treatments, but larger group sizes would require exploring these associations further.

CONCLUSIONS

Heat treatment of colostrum and hospital milk was more effective than UV treatment in decreasing bacterial counts. Colostral IgG concentrations were significantly reduced by heat and UV treatment. Lactoferrin concentrations were significantly reduced in treated colostrum, but not in UV treated hospital milk. Colostrum and hospital milk treatments were not associated with calf survivability, diarrhea, pneumonia or body weight gain. Parity, metritis of the dam, and calf birth weight were significantly associated with calf survivability. Parity, calf birth weight, and parturition assistance were significantly associated with the incidence of diarrhea. Metritis and retained placenta of the dam were significantly associated with calf pneumonia.

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CHAPTER 2

EFFECT OF AN INJECTABLE TRACE MINERAL SUPPLEMENT CONTAINING SELENIUM, COPPER, ZINC, AND MANGANESE ON IMMUNITY, HEALTH, AND GROWTH OF DAIRY CALVES

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ABSTRACT

The objective of this study was to evaluate the effect of 2 subcutaneous injections of a multimineral preparation, each containing 60 mg of zinc, 10 mg of manganese, 5 mg of selenium, and 15 mg of copper at 3 and 30 d after birth on immunity, health, and growth of dairy calves during the pre-weaning period. The study was conducted in upstate New York in 2 commercial dairy farms. A total of 790 Holstein heifer calves were randomly allocated at birth into 1 of 2 treatments: trace mineral supplement (TMS) treated or control. Blood samples were collected at 3, 14, and 35 d after birth to evaluate glutathione peroxidase (GPx) activity, super- oxide dismutase (SOD) activity, haptoglobin, and neutrophil and monocyte function. Incidence of diseases and average daily gain was evaluated in the first 50 d of life. At 14 d of life, TMS-treated calves had increased neutrophil activity compared with control calves. Moreover, TMS-treated calves had greater GPx activity on d 14 after birth than control calves.

The TMS treatment reduced the incidence of diarrhea (TMS = 41.7% vs. control = 49.7%) and combined incidence of pneumonia or otitis or both (TMS = 41.7% vs. control = 49.1%). Additionally, GPx was greater for calves diagnosed with otitis at d 35 after birth. However, calves diagnosed with pneumonia had decreased GPx activity at d 35 after birth. Serum SOD and haptoglobin concentrations were not affected by treatment or disease. More- over, no effects were observed on average daily gain and survivability between TMS-treated and control calves during the pre-weaning period. Supplementation with trace minerals at 3 and 30 d of life increased neutrophil function and GPx activity and reduced the incidence of health disorders.

INTRODUCTION

Dairy replacement rearing success or failure is de- pendent on several complex and interrelated factors. Newborn calf health and growth can be impaired by poor maternal health (Lundborg et al., 2003), dystocia (Lombard et al., 2007), colostrum deprivation (Weaver et al., 2000), and poor calf nutrition (Ollivett et al., 2012). In the modern dairy industry, calves are reared artificially and early nutritional programs have been extensively studied to improve their performance during the pre-weaning period (Soberon et al., 2012). The physiological processes of a livestock animal, including the immune system, can be largely influenced by the availability of nutrient and trace minerals that are essential for multiple biochemical processes, including immune response, cell replication, and skeletal development, and are particularly relevant for the newborn (Carroll and Forsberg, 2007).

Studies that evaluate trace mineral depletion or supplementation focus on critical times in calves' lives and evaluate the effects of factors such as transportation (Crookshank et al., 1979), stress (Galyean et al., 1999), and diseases (Orr et al., 1990). For adult cattle, stress during the transition period can affect trace mineral status (zinc) and immune suppression can lead to greater susceptibility to diseases (Enjalbert et al., 2006). Likewise, stress can affect trace mineral status in dairy calves. Nockels et al. (1993) reported that calves under induced stress (intramuscular injections of ACTH) reduced their ability to retain trace minerals. An injectable trace mineral solution containing Zn, Cu, Mn, and Se was reported to increase liver concentrations of Cu and Se for at least a 15-d period, and increased plasma Zn and Mn for several hours in Angus and Simmental steers (Pogge et al.,

2012).

Metabolic demands associated with stress and nutritional deficiency can lead to an increased production of reactive oxygen species (**ROS**; Sordillo and Aitken, 2009). When the production of ROS exceeds the antioxidant defense mechanisms present in the body, animals develop oxidative stress. Reactive oxygen species can initiate lipid peroxidation and cause cellular dam- age to tissues. Immune cells are particularly sensitive to oxidative stress because their membranes contain high concentrations of PUFA that are very susceptible to peroxidation, and produce large amounts of ROS when stimulated (Spears and Weiss, 2008). Several trace minerals are required for functioning of enzymes involved in the antioxidant defense system and may also affect immune cells via mechanisms distinct from antioxidant properties (Spears, 2000).

The role of early postnatal supplementation with injectable trace minerals on the immune response of new- born calves has not been investigated. Therefore, the objectives of this study were to evaluate the effect of supplementation of an injectable multimineral supplement containing Zn, Mn, Se, and Cu at 3 and 30 d after birth on peripheral blood neutrophil and lymphocyte function, oxidative stress markers, diseases (diarrhea, pneumonia, and otitis), and growth of Holstein heifer calves during the pre-weaning period.

MATERIALS AND METHODS

Farm, management, housing and feeding

The study was conducted in 2 commercial dairy farms located near Ithaca, New York, from February until December of 2012. Farms were chosen because of their relationship with Cornell University - Ambulatory Clinic. Farm A was milking approximately 2,800 cows and their calf rearing program was composed by two housing and feeding systems (group-pens fed ad libitum acidified non-sealable milk, and individual-pens fed pasteurized non-sealable milk). Farm B was milking approximately 1,600 cows and their calf rearing program was constituted of one housing type and feeding system with individual-pens fed pasteurized non-sealable milk.

Colostrum management was similar in both farms. Colostrum from primiparous and multiparous cows was pooled and refrigerated. Calves from farm A were fed approximately 4 L of raw colostrum within 4 h of birth at once by esophageal feeder (Oral Calf Feeder Bag with Probe, Jorvet). Calves from farm B were fed 2 L of raw colostrum within 2 hours and another 2 L approximately 6 hours later.

In farm A, calves were transported twice daily from the maternity area into two adjacent green-house type barn using two different rearing systems; group-pens and individual-pens. Group housing (n = 446) system was comprised of 15 group-pens for 20 calves. The group-pens of 70 square meters each were straw bedded on the top of a thin layer of dry composted manure. The barn was positive-pressure ventilated; pens were separated by stainless-steel gates. Calves were nipple-fed ad libitum acidified (Formic Acid 10% AgroChem Inc.) non-sealable milk (4.5 pH) until weaning (45 ± 5

d) based on milk reduction for 7 days.

Farm A (*n* = 194) and B (*n* = 150) had similar individual-pens set-ups in a green-house type barn, positive-ventilated comprised of 4 rows of 35 individual-pens; pens were 1.5 meter wide by 2 meter long isolated by flat plastic panels within each other bedded with a 0.5 meter deep gravel base that was covered with straw. Farm A and B had the same feeding system for calves allocated in individual-pens; each individual pen was provided with two 5 L plastic bucket; calves were fed a total of 6 L of pasteurized (72^{o}C for 15 seconds; T-600 and T-300 GoodNature Products Inc.) non-saleable milk divided equally twice a day; 0630h and 1700h. The same bucket was cleaned (manually using hot water with chlorinated detergent, followed by a warm rinse with water) and filled with water after each feeding; the second bucket was always filled with calf starter (starter composition **Table 2.1**). Calves were weaned at 45 (±5) days based on milk reduction for 7 days.

Table 2.1: Nutrient composition of calf starter diets for farms A and B.

	Starter Diet	
Composition	Farm A	Farm B
Dry Matter (%)	82.1	90.9
Crude Protein (% of DM)	19.8	22.9
Neutral Detergent Fiber (% of DM)	29.4	31.8
Acid Detergent Fiber (% of DM)	15.7	14.1
Total Digestible Nutrients (% of DM)	72	77
Calcium (% of DM)	0.99	0.94
Phosphorus (% of DM)	0.64	0.78
Magnesium (% of DM)	0.43	0.35
Potassium (% of DM)	1.4	1.01
Sodium (% of DM)	0.35	0.39
Sulfur (% of DM)	1.28	0.35
Iron (mg/kg)	398	258
Zinc (mg/kg)	65	111
Copper (mg/kg)	20	22
Manganese (mg/kg)	66	100

Study design, treatments, blood sampling and data collection

A total of 790 calves were randomly allocated into the 2 treatments: trace mineral supplement (TMS) treatment or control. Randomization was completed using the random number function in Excel software (Microsoft Corp., Redmond, WA). Calves allocated into the TMS treatment received two 1-mL subcutaneous injections (60 mg of Zn, 10 mg of Mn, 5 mg of Se, and 15 mg of Cu; Multimin North America Inc., Fort Collins, CO) at 3 and 30 d after birth. Calves allocated into the control group were left untreated. Body weight was measured weekly in both farms using a WayPig 15, 1.6-m (62-inch) digital scale (WayPig; Vittetoe Inc., Keota, IA) from birth until weaning, which occurred at 50 d of life.

Blood collection was performed via jugular venipuncture using an 18-gauge by 3.8-cm needle in 2 individual vacuum tubes: a 10-mL vacuum tube (Becton,

Dickinson and Co., Franklin Lakes) without anticoagulant for serum and an 8-mL heparinized vacuum tube (Becton, Dickinson and Co.) for plasma. Serum was harvested following centrifugation at 2,000 × g for 15 min at 4°C and plasma was harvested after centrifugation at 1,000 × g for 10 min at 4°C. Serum and plasma samples were stored at −80°C. Blood was sampled on d 3 (immediately before treatment administration), 14, and 35 after birth to evaluate glutathione peroxidase (GPx), superoxide dismutase (SOD), and haptoglobin (Hp). Additionally, serum IgG concentration was measured only on d 3.

A random subset of serum samples from 10 calves (5 for treatment) were sent to Veterinary Diagnostic Laboratory, Iowa State University (Ames) for analysis of Ca, Cu, Fe, K, Mg, Mn, Mo P, Se, and Zn. Blood mineral levels were measured at 3, 14, and 35 d after birth. The concentrations of serum trace elements were analyzed by an inductively coupled plasma mass spectrometry (ICP-MS) system (Varian/Bruker 820 ICP-MS; Bruker Corp., Fremont, CA) through separation of analyte ions from spectral interferences. Serum mineral concentrations were reported in milligrams per kilogram.

Serum IgG was measured using a radial immunodiffusion assay according to kit instructions (Bethyl Laboratories Inc., Montgomery, TX). Intraassay and interassay coefficients of variation for IgG were 3.2 and 3.6%, respectively. An SOD assay kit (Cayman Chemical Co., Ann Arbor, MI) was used, following the manufacturer's instructions for assaying SOD activity in plasma. The intraassay coefficient of variation for SOD was 3.7%. Likewise, a Cayman GPx assay kit was used, following the manufacturer's instructions to evaluate GPx activity. The intraassay coefficient of

variation for GPx was 7.2%. Serum Hp was evaluated following the method described by Makimura and Suzuki (1982). The intraassay coefficient of variation for Hp was 9.8%.

Additionally, a random subset of 228 calves (112 control and 126 TMS-treated calves) from one of the farms was sampled to evaluate neutrophil and monocyte function at d 14 after birth. Neutrophil and monocyte function was measured by the neutrophil and monocyte phagocytic activity using a PHAGOTEST kit (Orpegen Pharma GmbH, Heidelberg, Germany) containing fluorescein-labeled opsonized Escherichia coli (E. coli- FITC), following the manufacturer's instructions. Cells were analyzed with a FACSCalibur flow cytometer (Becton, Dickinson and Co.) using a 488-nm argon-ion laser. Ten thousands events were collected for each cell population (neutrophils or monocytes). The results are reported as the percentage of cells performing phagocytosis from the total number of cells in the granulocyte gate and as the increase mean of the green fluorescence of the gated cells.

Case definitions

The criterion to determine a case of pneumonia was the presence of two or more of the following clinical signs in a calf: cough, rectal temperature >39.5 °C, respiratory rate >40 breaths/minutes, increased cranioventral lung sounds or wheezes. Otitis was determined by observation of ear pain evidenced by head shaking and scratching at or rubbing ears, epiphora, ear droop, and signs of facial nerve paralysis, listlessness, and temperature >39.5 °C. The criteria do determine diarrhea was watery and fetid fecal consistency. Calves were recorded as diarrheic when diarrhea was

observed at least during 3 days throughout the study period.

Statistical Analyses

Descriptive statistics were performed using FREQ, MEANS, and UNIVARIATE procedures of SAS (SAS Institute). The initial body weight, IgG concentrations at day 3 after birth, percentage of calves born from primiparous cows, and assisted parturitions were evaluated to assure similar distribution between TMS and control calves within each farm and rearing systems.

For all health events (pneumonia, otitis, diarrhea, otitis and/or pneumonia, and mortality) calves were right censored at the end of the study period (50 days of life). To evaluate the effect of treatment on pneumonia, otitis, diarrhea, otitis and/or pneumonia, and mortality five multivariable mixed logistic regression models were fitted to the data using the GLIMMIX procedure of SAS. The variable described as "otitis and/or pneumonia" characterize calves that were affected with otitis, pneumonia, or both. The variables treatment group, serum IgG concentration (3 days of life), and birth weight were offered to all models. Additionally, the variable farm was included in all models as a random effect. Adjusted probabilities of pneumonia, otitis, diarrhea, otitis and/or pneumonia, and mortality were obtained using the LSMEANS statement.

The effect of treatment on ADG and leukocyte function was evaluated with general linear models using the MIXED procedure of SAS. Average daily gain was calculated subtracting the body weight at weaning (50 days of life) by the birth weight, and subsequently dividing by 50. Farm was used as a random effect and birth weight

and serum IgG concentration at 3 days of life were used as independent variables. Least square means and respective 95% confidence intervals were estimated for all categorical main effects.

Mixed general linear models were fitted to the data using the MIXED procedure of SAS to analyze the effect of treatment, pneumonia, otitis, and mortality on the repeated measures (3, 14, and 35 days of life) of GPx, SOD, and Hp. To control for repeated measures, the animal identification number (nested within farm) was included in all models as a random effect and serum IgG concentration (3 days of life), and birth weight were included as covariates. Separate models were used for treatment, pneumonia, otitis, and mortality to avoid multicollinearity. The interaction of each categorical variable and sampling time were offered to all models.

For all general linear models, the assumption that the residuals were normally distributed was assessed by visually evaluating the distribution plot of the Studentized residuals. Statistical significance was declared when P-value was ≤ 0.05 and statistical tendencies were declare when $0.05 < P\text{-value} \leq 0.10$.

RESULTS

Descriptive statistics

Average body weight (kg) at enrolment, serum IgG concentration at 3 days of life, percentage calves born to primiparous, percentage of assisted parturition, and number of calves enrolled in each calf farm by rearing system were not different for calves at TMS and control treatments (**Table 2.2**).

Table 2.2: The percentage of calves born from primiparous cows, assisted parturition, initial body weight (kg), and calves` serum IgG concentrations at day 3 after birth (g/L) were evaluated between TMS and control calves within the farms rearing systems. The LSMEANS and the respective SEM are presented for initial body weight and serum IgG concentration.

	Farm A (Group fed ad-libitum)			Farm A (Individual fed restricted)			Farm B (Individual fed restricted)		
	Control	TMS	*P*	Control	TMS	*P*	Control	TMS	*P*
n	230	215		100	95		70	80	
Calves born to primiparous	37%	37%	*0.9*	27%	27%	*0.9*	43%	45%	*0.8*
Assisted parturition	4%	5%	*0.7*	3%	5%	*0.4*	16%	18%	*0.8*
Initial weight	42.2 (0.4)	41.9 (0.4)	*0.4*	40.4 (0.5)	41.1 (0.5)	*0.4*	41.3 (0.6)	41.1 (0.6)	*0.8*
Serum IgG	23.5 (0.9)	24.0 (0.9)	*0.7*	22.4 (1.3)	23.2 (1.4)	*0.7*	26.7 (1.8)	25.7 (1.6)	*0.7*

TMS, trace mineral supplemented calves administered at 3 and 30 days of age
Initial weight, calves initial body weight (kg) at 3 days of life
Serum IgG, calves serum IgG (g/L) measured at 3 days of life, before first treatment

Disease incidence and average daily gain

The effect of treatment on adjusted incidences of mortality, diarrhea, otitis, pneumonia, and otitis and/or pneumonia is presented on table 2. Briefly, TMS group calves had lower (41.7%) incidence of diarrhea when compared to control group calves (49.1%; **Table 2.3**). Average weight gain was not significantly different between treatment groups (P = 0.49). The ADG for calves enrolled into the TMS group was; 778 g/day (95% C.I.; 729 – 826) and 789 g/day (740 – 837) for calves in the control group.

Table 2.3: A mixed logistic regression model was used to evaluate the effect of treatment on health events. Adjusted odds ratio and adjusted probabilities of pneumonia, otitis, diarrhea, otitis and/or pneumonia, and mortality were obtained using the LSMEANS statement. Adjusted odds ratios are presented with the respective 95% confidence intervals.

	Adjusted incidence %		Adjusted odds ratio (95% C.I.)	*P*-value
	TMS	Control		
Mortality	3.8%	2.7%	0.70 (0.30 – 1.58)	*0.39*
Diarrhea	41.7%	49.7%	1.38 (1.02 – 1.86)	*0.03*
Otitis	10.6%	13.2%	1.26 (0.82 – 2.01)	*0.30*
Pneumonia	35.2%	40.0%	1.23 (0.91 – 1.65)	*0.18*
Otitis and/or pneumonia	41.6%	49.1%	1.35 (1.00 – 1.83)	*0.04*

TMS, trace mineral supplemented calves administered at 3 and 30 days of age
Diarrhea, calves affected with diarrhea for at least 3 days
Otitis and/or pneumonia, this health event is characterized by calves that were affected with otitis, pneumonia, or both

Blood leukocyte function

Peripheral blood leukocyte function was greater for TMS calves than for control calves at 14 days after birth (**Figure 2.1**). Briefly, TMS calves had increased neutrophils activity showing an increased mean fluorescence, i.e. number of ingested bacteria by neutrophils ($P = 0.05$) and percentage of neutrophils that performed

phagocytosis ($P < 0.01$) when compared with control calves.

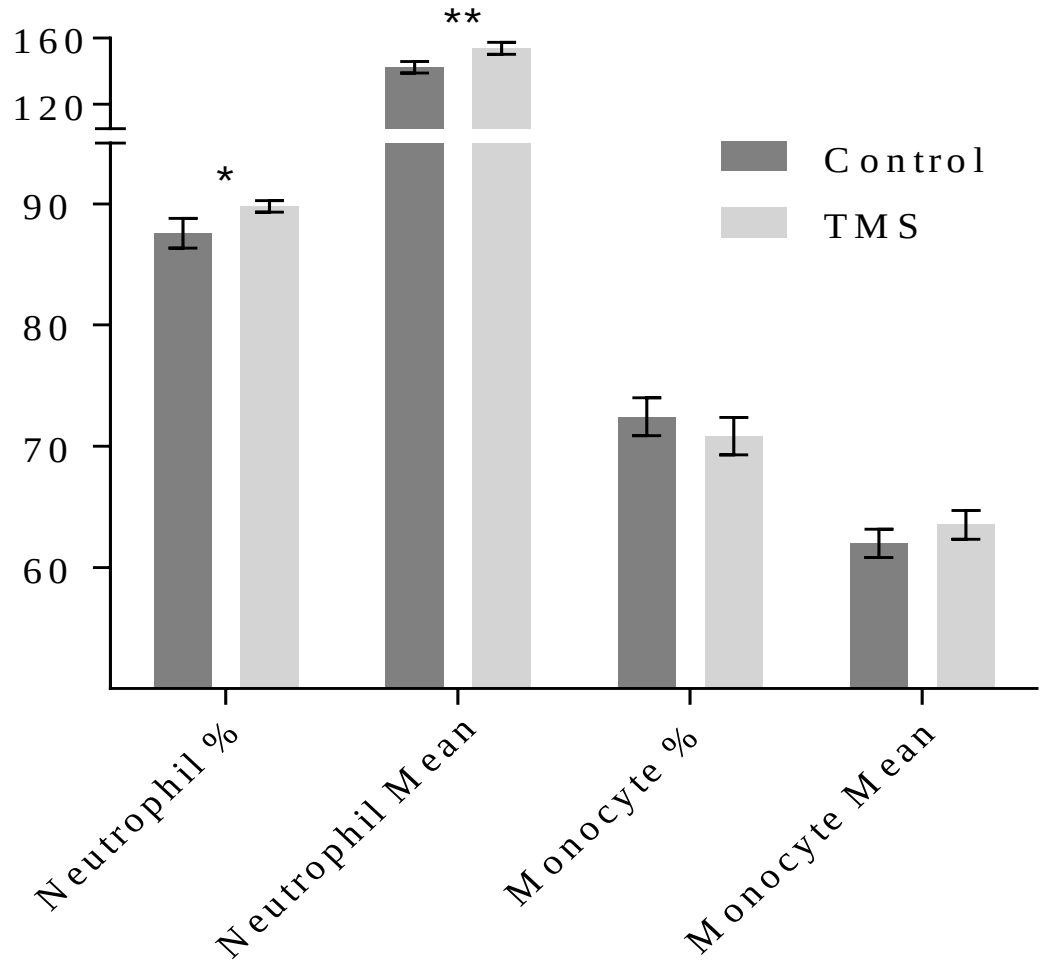

Figure 2.1: Effect of trace mineral injections (TMS; 3 and 30 days of life) on the percentage and mean fluorescence intensity (reflecting ingested bacteria) of neutrophils and monocytes assessed on blood samples collected at 14 days. The dark-gray bars represent calves on the control group and light-gray bars calves on TMS group. Trace mineral supplemented calves had greater overall neutrophil mean when compared to control calves. Error bar represent the standard error of the mean. * indicates *P* – value between 0.1 and 0.05, ** indicates *P* – value less than 0.05.

Additionally, blood leukocyte function was not different for calves that were recorded with pneumonia, otitis, diarrhea, and calves that died before the end of the study period when compared with healthy and calves that survival during the study period (**Figure 2.2**).

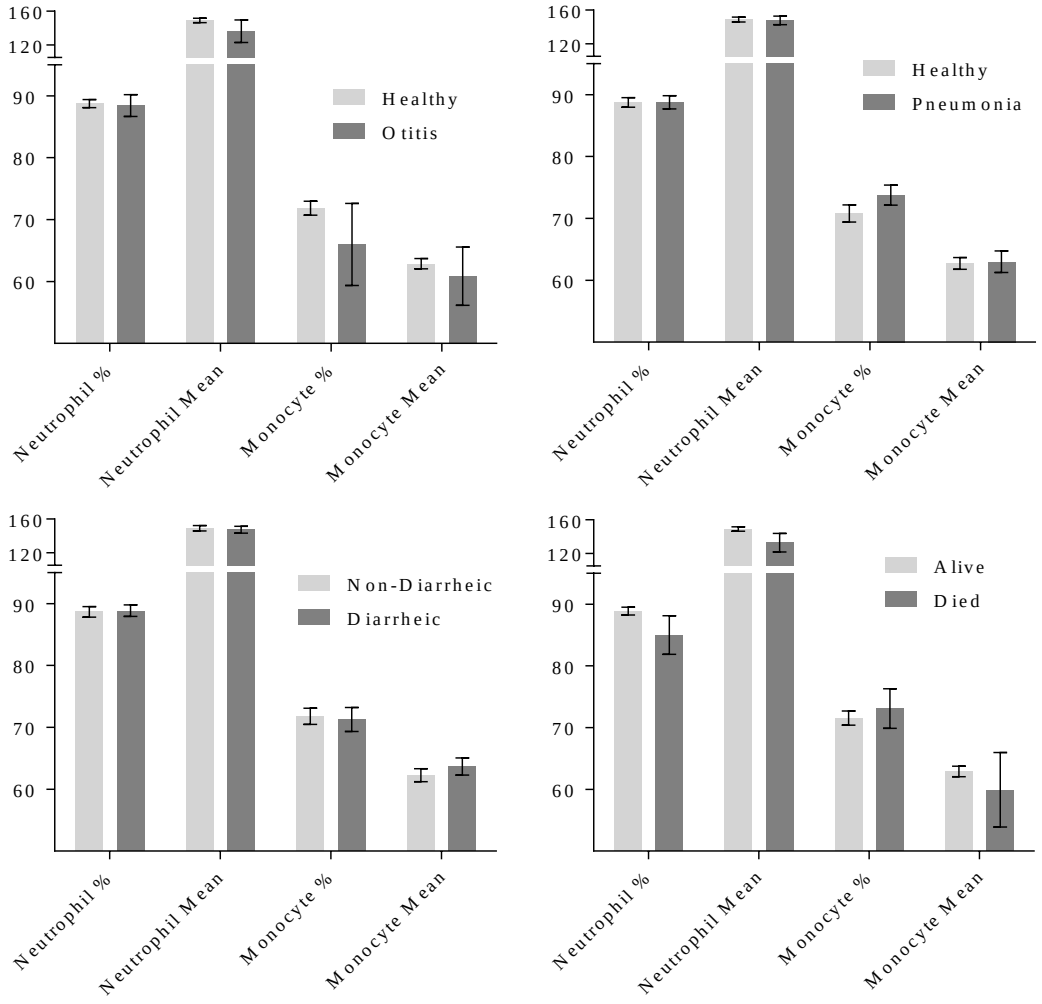

Figure 2.2: Percentage and mean fluorescence intensity (reflecting ingested bacteria) of neutrophils and monocytes assessed on blood samples collected at 14 days of life. The dark-gray bars represent calves that were affected with otitis, pneumonia, diarrhea, and calves that died and light-gray bars represent calves that were not affected with disease and survival until the end of the study (50 days). Error bar represent the standard error of the mean. * indicates *P* – value between 0.1 and 0.05, ** indicates *P* – value less than 0.05.

Oxidative stress and acute phase protein markers

Calves supplemented with trace minerals had increased GPx when compared with control calves at day 14 after birth. Interestingly, calves diagnosed with pneumonia had a significantly lower glutathione peroxidase activity at day 35 after birth when compared with healthy calves (**Figure 2.3**). There were no effects of treatment on serum SOD activity and Hp optic density at days 3, 14, and 35 after birth (Figure 4 and 5). Moreover, there were no effects of disease on calves` serum SOD activity and haptoglobin optic density (**Figure 2.4 and 2.5**).

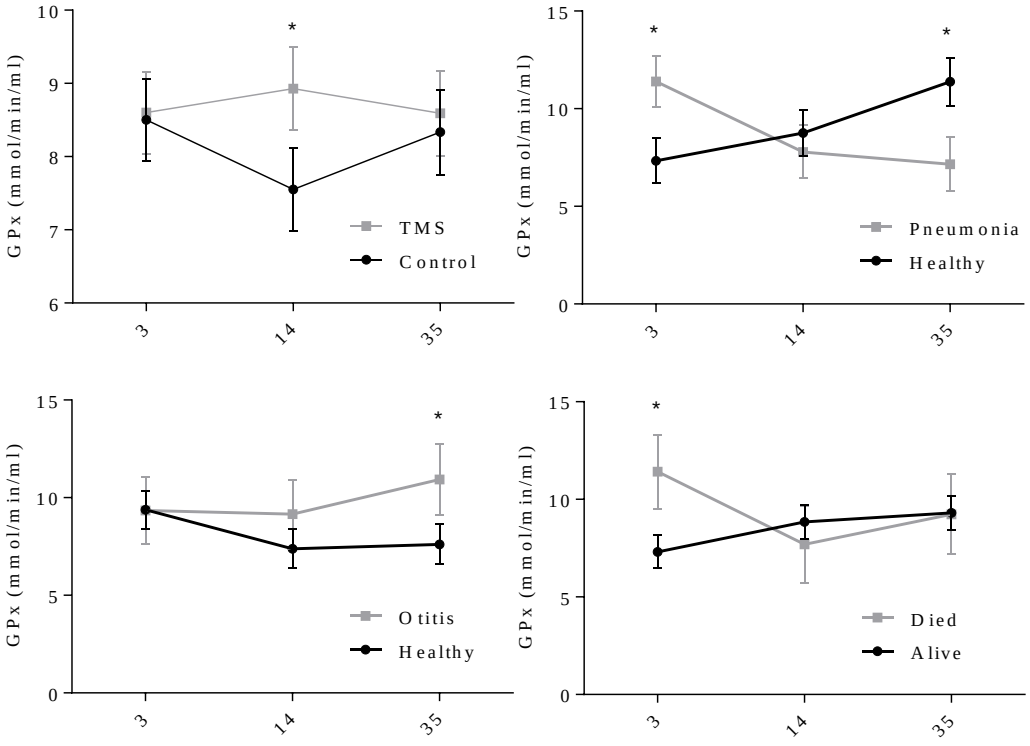

Figure 2.3: Plasma glutathione peroxidase (GPx) activity (nmol/min/mL) was assessed on days 3, 14, and 35 of the calves` life. The light-gray lines represent the effect of trace mineral supplemented calves (TMS vs. control calves), pneumonia, otitis, and calves that survival until the end of the study period (50 days) on the repeated measurements of GPx. Error bar represents the standard error of the mean. * indicates *P* – value less than 0.05.

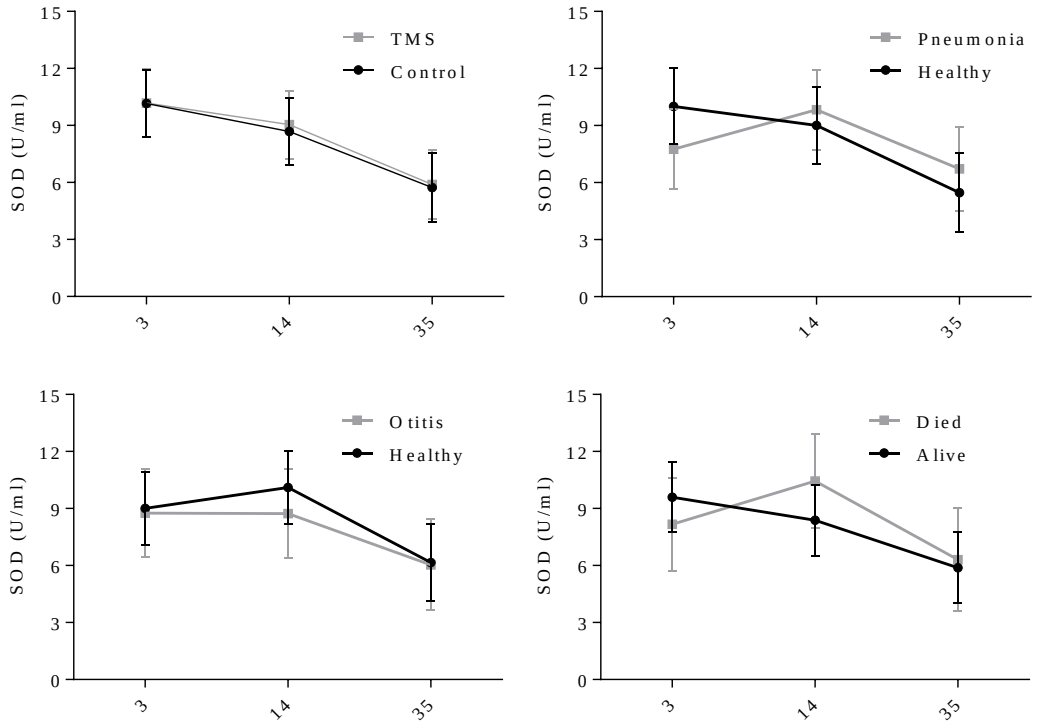

Figure 2.4: Serum superoxide dismutase (SOD) activity (U/mL) was assessed on days 3, 14, and 35 of calves` life. The light-gray lines represent the effect of treatment, pneumonia, otitis, and calves that survival until the end of the study period (50 days) on the repeated measurements of SOD. No effect was observed on SOD activity for trace mineral treated calves (TMS vs. control calves), pneumonic calves (vs. healthy), calves diagnosed with otitis (vs. healthy), and calves that survival until the end of the study period (vs. calves that died). Error bar represents the standard error of the mean.

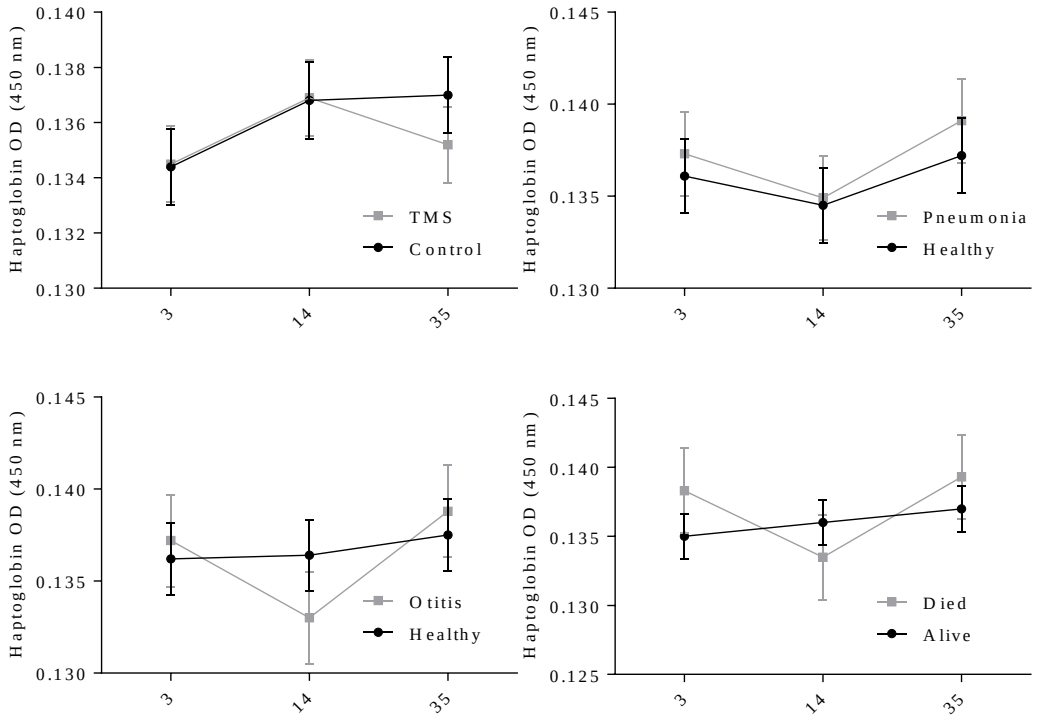

Figure 2.5: Serum haptoglobin (Hp) optic density (OD) was evaluated on days 3, 14, and 35 of calves` life. The light-gray lines represent the effect of treatment, pneumonia, otitis, and calves that survival until the end of the study period (50 days) on the repeated measurements of SOD. No effect was observed on Hp OD for trace mineral treated calves (vs. control calves), pneumonic calves (vs. healthy), calves diagnosed with otitis (vs. healthy), and calves that survival until the end of the study period (vs. calves that died). Error bar represents the standard error of the mean.

DISCUSSION

As anticipated, supplementation with trace minerals at early postnatal life was beneficial to calves immunity and oxidative stress status. Calves supplemented with trace minerals had improved leukocyte function increasing its ability to perform phagocytosis and improved glutathione peroxidase activity. Trace minerals such as selenium, zinc and copper are required for functioning of enzymes involved in the antioxidant defense system and may also affect immune cells through mechanisms distinct from antioxidant properties. Selenium is an essential component of glutathione peroxidase enzymes destroying hydrogen peroxide and lipid hydroperoxides (Rotruck et al., 1973).

Copper and zinc form the Cu–Zn superoxide dismutase (SOD), which is responsible for dismutation of superoxide radicals to hydrogen peroxide in the cytosol (Halliwell and Gutteridge, 1999). Zinc also induces synthesis of a metal binding protein that may scavenge hydroxide radicals called metallothionein (Prasad, 2004). In addition to an antioxidant role, zinc may affect immunity via its important role in cell replication and proliferation (Spears and Weiss, 2008). The increased activity of enzyme glutathione peroxidase suggests that the trace mineral supplementation can effectively reduce oxidative stress in calves in the first two weeks of life. Considering the particular immune cells sensitivity to oxidative stress, it is possible that improved phagocytosis ability of leukocytes was a function of the enhanced antioxidant status provided by the trace mineral supplemented.

Phagocytosis is one of the most important functions of the neutrophils playing a critical role on their ability to recognize and eliminate foreign and infected cells. The

effect of Se supplementation was already described to improve phagocytosis in white blood cells population (Hogan et al., 1990). Moreover, Suwanpanya et al. (2007) reported an increase in the neutrophils phagocytic activity on *Staphylococcus aureus* of heifers dietary supplemented with 3mg Se and 4,000 IU vitamin E per day. In the current study, 14 days old calves` peripheral blood neutrophils were tested against opsonized *Escherichia coli* and the results indicated significant improvement in the phagocytic activity for calves supplemented with 1mg of selenium at 3 days of life when compare with not supplemented calves. Although trace mineral supplementation significantly increased glutathione peroxidase activity at 14 days of age in this study, no effect of treatment was observed at 35 days of life. Interestingly, the current study reported a decreased glutathione peroxidase activity for calves diagnosed with pneumonia and an increased activity for calves diagnosed with otitis. Cemek et. al (2006), report similar results in an investigation of antioxidant status in children with acute pneumonia where glutathione peroxidase activity was reduced in affected children compared to non-affected. Additionally, another study using human infants found a decreased level in plasma GPx in 57 patients with pneumonia when compared to 87 controls (Cui et al., 1997).

Bonham et al., (2002) suggested that neutrophils function might be a valuable biomarker for copper status. However, in the current study supplemental trace minerals did not influence superoxide dismutase, the antioxidant enzyme that uses copper and zinc to be synthesized. Likewise, haptoglobin a marker of acute phase protein was not affect by treatment. Acute phase proteins are well described as quantifiable indicators of inflammation of infection in adult cattle. However, there are

some factors that can significantly change acute phase protein concentration in neonatal calves; stress of birth, microbial challenge, and intake of colostral cytokines.

The beneficial effects of trace mineral supplementation on immune function were ultimately reflected on lower incidence of diarrhea. Oral Zn supplementation was reported to reduce the incidence of diarrhea among children in developing countries (Funchs 1998; Hambridge et al., 2000). Furthermore, the effectiveness of zinc in children diarrhea was related to improvement in the enzymatic functions of the brush borders, and enhancement of the intestinal mucosa repair (Sazawal et al., 1995: Strand et al., 2002).

However, the benefits of trace mineral supplementation herein reported did not translated in improved average daily gain. Clearly, within a calf rearing program a multitude of factors can play a role on growth and health status of calves. Factors such as individual measurements of DMI, milk consumption, and most important biological levels of trace minerals throughout the first 60 days of life may contribute to the results herein presented. Although, leukocyte function and oxidative stress status were improved in TMS calves the first two weeks of life, the absence of effects on average daily gain, pneumonia, otitis and survivability for TMS calves and the significant but punctual effect of TMS on neutrophils function should be carefully examined following the data used for these inferences. Future studies should consider individual DMI, milk consumption and biological levels of trace minerals in the pre-weaned period as potential factors to improve health and growth for dairy calves.

CONCLUSIONS

In conclusion, in this study trace minerals supplementation improved leukocyte function and oxidative stress status of dairy calves in first two weeks after birth. However, these benefits did not translate into improved growth performance and health status for dairy calves in the first 60 days of life. Some critical factors such as individual DMI, milk consumption, and biological levels of trace minerals may shed light on critical points for a proper supplementation of trace minerals that results in benefits for growth and performance. Therefore, the improved immunological status of dairy calves supplemented with trace minerals uncoupled with growth and health performance warrants further investigation.

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CHAPTER 3

EFFECT OF CROFELEMER EXTRACT ON SEVERITY AND CONSISTENCY OF EXPERIMENTALLY INDUCED ENTEROTOXIGENIC ESCHERICHIA COLI DIARRHEA IN NEWBORN HOLSTEIN CALVES

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ABSTRACT

The objective of this study was to evaluate the effect of standardized botanical extract of *Croton lechleri* named crofelemer extract, on fecal dry matter and fecal scores on diarrheic newborn Holstein bull calves induced by enterotoxigenic *Escherichia coli* (ETEC). A double-blinded randomized clinical trial was performed, 60 newborn Holstein bull calves were clean caught and transported to an isolation facility where calves were individually housed and randomly assigned to one of three treatment groups; placebo (CTR), enteric-coated formulation of crofelemer extract (ECROF), and non-enteric coated formulation of crofelemer extract (CROF). Diarrhea was induced at first feeding with an inoculum of the ETEC (ATCC - 31616) administered with a third of the recommended dose of a colostrum replacer. All calves enrolled in this study received treatments starting on the second feeding (diarrhea onset) and treatments were administered before feeding time (600h and 1600h) for 6 feedings consecutively. All calves in this study had failure of passive transfer. The only cause of death in this study was due to septicemia, accounting for one death out of each treatment group. All the calves were examined twice daily, within 2 hours after feeding, from day 1 (pre-challenge) until day 10, on day 15, and a last examination on day 25 of the calves` life. Five parameters were evaluated during each examination; rectal temperature, clinical assessment of dehydration status, fecal scores, attitude, and appetite. No differences were observed between treatment groups for rectal temperature, attitude, and appetite. Fecal dry matter was analyzed as pre-challenge fecal dry matter, dry matter during treatment, and fecal dry matter after treatment cessation. No difference in pre-challenge fecal dry matter was observed and

pre-challenge fecal dry matter was used as a covariate in the models. Fecal dry matter during treatment was significantly higher for ECROF calves when compared to control calves and CROF calves. Additionally, ECROF fecal dry matter after treatment cessation had a statistical tendency to be higher when compared to control calves. Together, these results suggest that enteric coated formulation of the standardized crofelemer extract, a natural-product with anti-secretory properties, can significantly increase fecal dry matter of neonatal calves with experimentally induced enterotoxigenic *Escherichia coli* diarrhea. More research is needed to test the efficacy of enteric coated crofelemer on incidence and severity of secretory diarrhea on calves under natural challenge conditions.

INTRODUCTION

Considerable economic losses may be incurred from neonatal diseases during calf rearing. In 2010, diarrhea (18%) and pneumonia (16%) were the most common disorders affecting pre-weaned heifers reported by the National Animal Health Monitoring System (NAHMS, 2011). Diarrhea is a multifactorial disease that can be caused by infectious and non-infectious factors (Walker et al., 1998; O'Handley et al., 1999). The risk factors known to be associated with diarrhea are; management (environmental condition), nutritional state, immune status, and pathogen exposure (Klein-Jöbstl et al., 2014; Al Mawly et al., 2015). Enteropathogens such as viruses, bacterial, and protozoa are often identified as etiological agents in calf diarrhea. The most common enteropathogens described in the literature include *Escherichia coli*, *Salmonella spp*., *Cryptosporidium*, and rotaviruses (Moon et al., 1978; O'Handley et al., 1999; Gulliksen et al., 2009; da Silva Medeiros et al., 2015).

Enterotoxigenic *E.coli* and neonatal diarrhea have been extensively studied in the last 4 decades (Bywater and Logan, 1974; Radostits, 1975; Acres, 1985). Much has been done to prevent early life contamination with enteropathogens; minimal dam-calf contact, colostrum pasteurization, and disease control practices (Moon and Bunn, 1993; Sandra, 2008; Naylor, 2009). In spite of efforts controlling neonatal diarrhea, it still remains as a major concern in the beef and dairy industry, with high impact on animal welfare and profitability (NAHMS, 2011; Hughes, 2013).

There are many important aspects on prevention and treatment of diarrhea due *Escherichia coli*. Vaccination of the cows before parturition can effectively provide antibodies against strains of enterotoxigenic *Escherichia coli* (ETEC) (Frantz et al.,

1987). The vaccination can also prevent neonatal diarrhea (Nagy, 1980). Antibiotics have been used as treatment against diarrhea caused by *Escherichia coli* (Sunderland et al., 2003). However, the use of antibiotics is under scrutiny due to concerns of bacterial resistance that could impact human health. Alternative therapeutic treatments for diarrhea have become more common. Studies have been conducted testing the efficacy of organic and inorganic zinc, probiotics, and even bacteriophages targeting enteropathogens for treatment of diarrhea in calves (Muscato et al., 2002; Bicalho et al., 2012; Glover et al., 2013).

The FDA recently approved crofelemer, a polyphenolic molecule isolated from the latex of the plant species *Croton lechleri* of the family *Euphorbiaceae* indicated for idiopathic HIV-associated secretory diarrhea. Crofelemer has been studied for its antisecretory actions that involve the inhibition of two distinct chloride channels on the luminal membrane of the intestine; cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channel (CaCC) (Tradtrantip et al., 2010). Cystic fibrosis transmembrane conductance regulator is expressed at the apical membrane of enterocytes and plays an important role in the intestinal physiology. Enterotoxigenic *Escherichia coli*, secretes bacterial toxins that leads to the activation of CFTR and CaCC, consequently leading to chloride secretion and intestinal fluid hyper secretion (Thiagarajah and Verkman, 2013).

To the best of the authors' knowledge, the efficacy of crofelemer extract as an antidiarrheal drug has not been previously evaluated in a neonatal bovine secretory diarrhea model. Here, we investigate the anti-secretory potential of crofelemer extract in newborn Holstein bull calves challenged with ETEC in the first day of life. The

objective of this study was to evaluate the effect of two formulations of crofelemer extract on diarrhea severity and consistency of experimentally-induced diarrhea in newborn Holstein calves in the first five days of life. As a second objective, the overall health status and performance of the calves were evaluated until 25 days of life.

MATERIALS AND METHODS

Experimental design

This experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Cornell University (Protocol number 2013-0075). Sample size was based on a fecal dry matter measured on a pilot study. Using a mean difference between groups of 1.4, a treatment group standard deviation of 1.2 and a placebo group standard deviation of 1.7, with a treatment group ratio of 1, assuming a desired type I error rate of 5%, a power of 80%, a sample size of 18 calves per group was calculated. As 10% of mortality was anticipated, a total of 20 calves were enrolled in this study.

The study design was a double-blinded randomized clinical trial. Randomization was performed a priori to beginning of the trial using Excel random function to create a balanced number of calves per treatment group (n = 20 calves per group). Data collection blindness was simplified by treatment tablets similarities, tablets were manufactured to have same appearance (same color and size) and were inodorous. A total of 60 Holstein bull calves from one commercial dairy farm (King Ferry, New York) were enrolled in the study. Calves were clean caught at parturition (minimizing animal contact between dam/calf and calf/maternity bed) and within 2

hours transported to an isolation facility for research animals at Cornell University. All the calves were enrolled in the study within 3 hours after birth. Transportation and vehicle cleaning procedure were performed accordingly to the Animal Care and Use Procedure. Briefly, calves were transported using an adapted cargo-van; calves were kept inside individual crates with proper ventilation, and transported directly from the farm to the research facility. Cleaning and sanitization of the vehicle was performed 30 minutes before and immediately after transportation.

Calves were individually housed in 16 square meter rooms with controlled temperature and humidity. Each isolation room had an individual inner-room, containing all the necessary instrumentation for feeding, treatments, weighing, cleaning, and data collection. No equipment was shared between calves. Additionally, calves were unable to have any contact with other calves and/or outside areas. Cleaning and sanitation of bottles, nipples, and buckets used to held milk (before and after each feeding), water (once daily), and calf starter (twice weekly) were manually performed by a three step cleaning procedure. The three step cleaning procedure consists in rinsing all the equipment with lukewarm water, scrubbing a mixture of hot water and alkaline detergent solution, and finally rinsing in chlorinated water.

Calves were fed antibiotic-free milk replacer (Nutrablend 22/20, Ranch-Way) by bottle on a 10% body weight daily basis twice a day (0600h and 1800h) during the first 3 days of life. Calves were gradually removed from bottles and encouraged to drink from the bucket. Water was available ad-libitum from day 1 until the end of the study. All calves were kept in the study until 25 days of life with ad-libitum access to calf starter (Calf starter 18% CP, DuMOR) starting on the 7^{th} day of life.

Escherichia coli inoculum and challenge

All the calves were challenged using an Enterotoxigenic Escherichia Coli (ETEC) serotype O9:K35:K99 (ATCC #31616). The ETEC inoculum used in this study was prepared two weeks previously to the beginning of the trial. Standard ETEC ATCC bacteria activation was performed using trypticase soy broth (Trypticase, Becton, Dickinson and Co.) to grow the bacteria for 8 hours and then on Luria-Bertani agar (Difco LB Agar, Becton, Dickinson and Co.) for 18 hours at 37°C. The bacteria was suspended in phosphate-buffered saline with 10% dimethyl sulfoxide and stored in 10 ml aliquots at -70^{o}C. The mean inoculum titer was 4 x 10^{10} colony-forming units per 10 ml.

All the calves were challenged at the research facility within 6 hours of life. A mixture of freshly prepared 1L of colostrum replacer containing 157g of colostrum replacer powder, approximately 35g of IgG (Calf Colostrum Replacer, Land O Lakes) was mixed with 10 ml of thawed ETEC inoculum. The mixture was administered within 1 minute after preparation for each calf via esophageal feeder. Feeder were used only once per calf and properly discarded (Oral Calf Feeder, Jorvet).

Treatment administration and data collection

Calves were assigned into 1 of 3 treatment groups; control (CTR; n = 20), enteric coated formulation of crofelemer extract (ECROF; n = 20), and non-enteric coated formulation of crofelemer extract (CROF; n = 20). The treatments were administered before each meal for 3 days consecutively (total of 6 treatments per calf) starting on the first feeding after challenge.

Only one trained caretaker was feeding the animals during the whole study period and only one member of the study group was responsible for the challenge, treatment administration and data collection. Calves were weighed at birth, 10, 15, and 25 days of life using a portable scale. Blood samples were collected via jugular venipuncture using an 18-gauge by 3.8-cm needle in an 8-mL vacuum tube (Becton, Dickinson and Co.) without anticoagulant for serum. Blood samples were collected between the morning and afternoon feedings; the first blood sample (baseline) was taken immediately before challenge and 4 subsequent blood samples were taken daily.

Serum was harvested following centrifugation at 2,000 × *g* for 15 minutes at 4°C. Serum IgG was measured using the second blood collection (2^{nd} day of life) using a radial immunodiffusion assay according to kit instructions (Bethyl Laboratories Inc.). Total solids (TS) were evaluated in serum samples using an optical refractometer.

All the calves were examined twice daily, within 2 hours after feeding, from day 1 (pre-challenge) until day 10, on day 15, and a last examination on day 25 of life. Five parameters were evaluated during each examination, rectal temperature, dehydration status, fecal scores, attitude, and appetite.

Dehydration status, attitude, and appetite scores were based on a numerical scale as follows; dehydration status: 0 = normal, eyes are bright and skin feels pliable; 1 = mild dehydration, slight loss of skin elasticity, skin tent < 3 seconds, eyes not recessed into orbit; 2 = moderate dehydration, skin tent > 3 seconds but < 10 seconds, eyes slightly recessed into orbit; 3 = severe dehydration, skin tent > 10 seconds, eyes markedly recessed into orbit. Attitude: 0 = alert, 1 = depressed, 2 = non-responsive.

Appetite: 0 = normal, 1 = consuming less than half of the meal, 2 = consuming less than 25% of the meal, 3 = not consuming. Fecal scores were a 5 point scale score (**Figure 3.1**) based on fecal consistency.

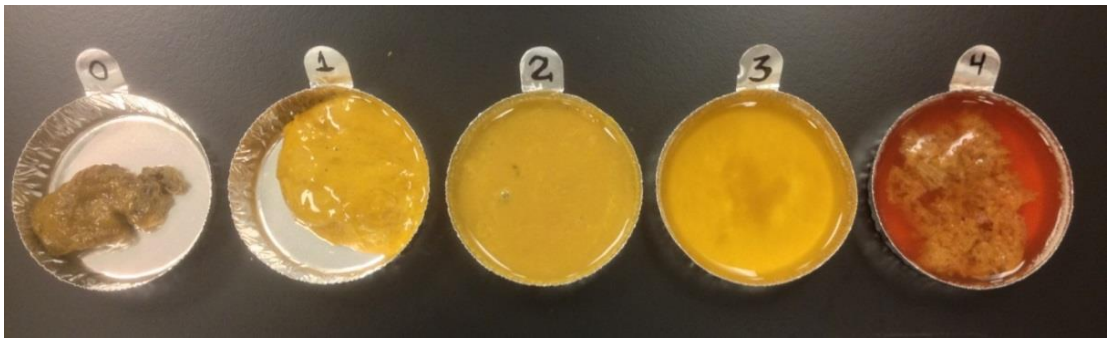

Figure 3.1: Fecal scores were based on diarrhea severity and the picture represents the 5 point scale used to visually assess calf diarrhea on milk fed calves. From the left to the right; 0 = formed feces with normal color, 1 = pasty with normal color, 2 = liquid with normal color, 3 = watery with normal color, 4 = watery with abnormal color.

Fecal samples were also collected (20ml plastic vials) during each clinical evaluation via digital stimulation on the calf`s rectum to evaluate percentage of dry matter of the feces. Fecal dry matter was determined as described by Bellosa et al., (2011). Briefly, 5 to 20 g of the sample was weighed on a precision digital scale (6202-1S, Sartorius) and then dried at 108 ^{o}C for 24 hours using an oven (Model 10 Lab Oven, Quincy Lab) and re-weighed immediately to determine the percent dry matter.

Fluid therapy

As a rescue treatment, an oral electrolyte (Hydralyte, Lloyd Inc.) was fed via bottle as an extra meal administered in between meal hours to calves that had a fecal

score ≥ 3 and dehydration score ≥ 2. Intravenous fluid therapy using isotonic sodium bicarbonate was administered by jugular vein catheter (70 mL/kg/hour) if calves were unable to stand, presenting very weak or no suckle reflex, and dehydration score ≥ 2.

Statistical Analyses

A total of 24 examinations/samples were collected per calf in this study. For the continuous data collected on fecal dry matter (FDM) and rectal temperature, calf samples were used as pre-challenge (defined as the first examination/sample before the challenge), during treatment days (defined as the calf average data collected during 3 days of treatments; 2nd to the 7th examination/sample), and after treatment cessation (defined as the calf average data collected after treatment cessation; 8th to the 24th examination/sample). Data on FDM and temperature was averaged within calf.

Statistical analyses were performed using JMP 10 (SAS Institute Inc.). An ANOVA was used to evaluate serum immunoglobulin (IgG). Blood samples from the second collection (post-challenge) were used to measure serum immunoglobulin-G. Intraassay and interassay coefficients of variation for IgG were 3.2 and 3.6%, respectively.

To evaluate the effect of treatment on the following dichotomized clinical outcomes; diarrhea (fecal score ≥ 3), depression (attitude score ≥ 1), hypophagia (appetite score ≥ 1), dehydration (dehydration score ≥ 2), and oral electrolyte administration (yes or no), Fisher`s exact test was used to compare the percentage of calves affected in each treatment group against control calves. Additionally, the number of events (diarrhea, depression, hypophagia, dehydration, and oral electrolyte

administration) recorded during treatments days and after treatment cessation, was evaluated using ANOVA comparing the treatment groups against the control group.

A mixed general linear (MGL) model was used to analyze the effect of treatment on rectal temperatures. Initial rectal temperature (pre-challenge) was used as a covariate in the MGL models to evaluate rectal temperature.

A mixed general linear (MGL) models were used to analyze the effect of treatment on average daily weight gain (ADG). Calves enrolled in the ECROF group had a numerically smaller average birth weight at enrollment when compare to calves on CTR and CROF groups ($P = 0.14$), for that reason, birth weight was used as a covariate in the MGL models to evaluate ADG. Average daily weight gain was calculated on days 10, 15 and 25 of the calves` life by subtracting the birth weight from the weight at 10, 15 and 25 days of life; this variables were then used as the outcome variable for the MGL models.

A similar mixed general linear (MGL) model was used to analyze the effect of treatment on serum total solids (TS). The data consists on TS measured at pre-challenge (day 0), day 1, day 2, day 3, and day 4 of calves' life. Pre-challenge TS measurement was used as covariate in this model. To control for repeated measures the animal identification number was added in the model as a random effect.

For all general linear mixed models, the assumption that the residuals were normally distributed was assessed by visually evaluating the distribution plot of the Studentized residuals. Statistical significance was declared when P was ≤ 0.05 and statistical tendencies were declare when $0.05 < P \leq 0.10$. Results are presented as least square means and standard error of the mean.

RESULTS

Three calves, one in each group, were euthanized within 72 hours of life due to signs of bacteremia. Euthanasia (penetrating captive-bolt) was carried out by trained personnel. All data collected from these calves were not used in the analysis. Those calves were the only calves in the present study that needed intravenous fluid therapy (IV) and as a result, no IV data was used. All euthanized calves were submitted to the Animal Health Diagnostic Center (Cornell University) for a postmortem examination and sepsis was confirmed.

No statistical differences were observed for 24hr serum IgG (mg/ml) between treatment groups; 2.80 (±0.29), 3.00 (±0.28), and 2.85 (±0.27) for CTR, ECROF, and CROF groups respectively (*P = 0.87*).

Pre-challenged fecal dry matter (**Figure 3.2**) was not significantly different between treatment groups; 21.41% (±1.63), 20.67% (±1.55), and 21.25% (±1.64) for CTR, ECRO, and CROF respectively (*P = 0.96*). Average fecal dry matter was significantly higher for ECROF 15.45% (±1.55) calves during treatment days when compared to control calves 11.15% (±1.63) and when compared to CROF calves 11.16% (±1.64). However, no difference was observed between CROF and control calves (*P = 0.96*). ECROF calves had a statistical tendency to have higher average fecal dry matter when compared to control calves (*P = 0.08*) following cessation of treatment. However, no significant difference in fecal dry matter after treatment cessation was observed between CROF and ECROF (*P = 0.73*) nor CROF and CTR (*P = 0.16*).

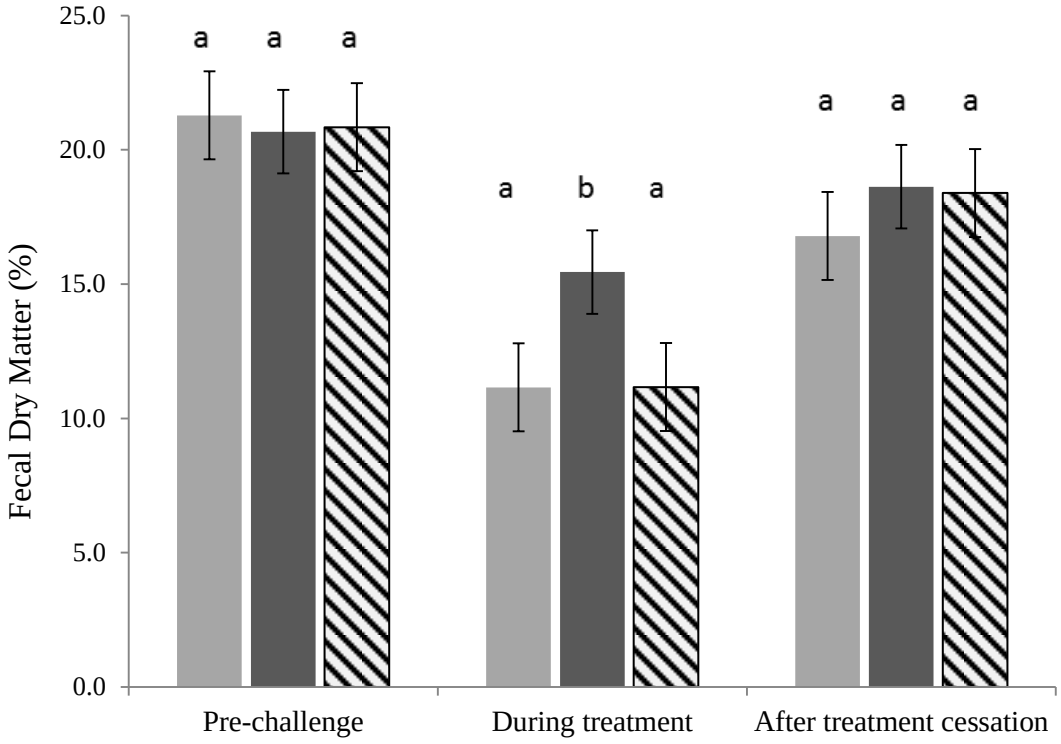

Figure 3.2: Effect of treatment on calves` fecal dry matter content (percentage of dry matter) measured on; pre-challenge, during treatment days, and after treatment cessation. Light gray bar represents control (CTR), dark gray bar represents enteric-coated group (ECRO), and downward diagonal bar represents non-enteric coated group (CROF). Error bars are presented as standard error of the mean. Data are presented as means and standard error of the mean. Different letters within period (pre-challenge, during treatment days, and after treatment cessation) represent statistical differences ($P < 0.05$).

Serum harvested from 5 blood samples per calf were used to measure total solids (TS, **Figure 3.3**) using an optical refractometer. There were no baseline (day 0) differences in TS between treatment groups (*P = 0.54*). There was a statistical tendency on day 1 collection in serum TS to be lower for calves in ECROF group when compared to CTR group (*P = 0.06*). No differences were found between treatment groups on day 2. Serum total solids were significantly higher for CTR calves when compared to ECROF calves (*P = 0.03*) and when compared to CROF calves (*P = 0.05*) on day 3, but no differences between ECROF and CROF groups. Additionally, serum total solids were significantly higher on day 4 collection for calves in CTR group when compared to ECROF (*P = 0.05*) and CROF (*P = 0.02*).

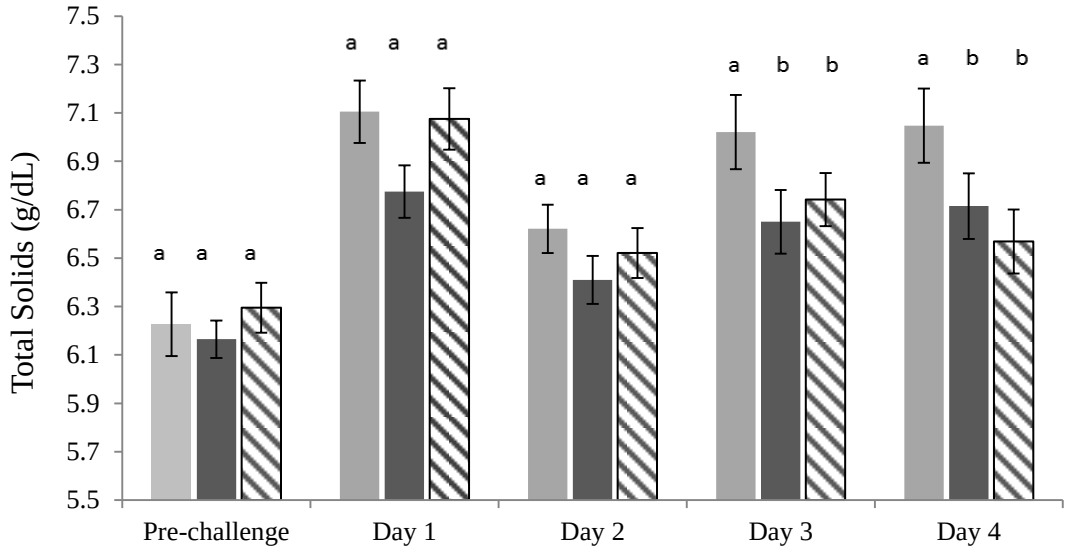

Figure 3.3: Effect of treatment on calves` serum total solids measured on blood samples collected 5 times at; pre-challenge (day 0), day 1, day 2, day 3, and day 4 of calves' life. Light gray bar represents control (CTR), dark gray bar represents enteric-coated group (ECROF), and downward diagonal bar represents non-enteric coated group (CROF). Error bars are presented as standard error of the mean. Data are presented as means and standard error of the mean. Different letters within period represent statistical differences (*P < 0.05*).

Diarrhea incidence pre-challenged was similar between treatment groups; 5.6% for CTR, ECROF, and CROF (*P = 1*). During treatment days, calves in the control group had 68.4% diarrhea incidence while calves in the CROF group had 84.2% (*P* = 0.44) and calves in the ECROF group had 57.9% (*P* = 0.74). Additionally, no differences were found between the mean duration of diarrhea between control calves, calves in the CROF group, and calves in the ECROF groups. However, after treatment cessation control calves had 57.9% incidence of diarrhea while only 15.8% of the calves in the ECROF group were diarrheic (*P* = 0.007) and no differences were found between the control group and calves in the CROF group. Moreover, diarrhea duration was significantly lower for calves in the ECROF group when compared to calves in the control group (*P* = 0.02).

The percentage of calves with decreased appetite (hypophagia), and dehydration were not found to be significantly different between treatment groups at the pre-challenge, during treatment days, nor after treatment cessation between treatment groups (**Table 3.1**).

In addition to the clinical evaluation, rectal temperature was not significantly different between treatment groups before challenge; 38.9^{o}C (±0.07), 38.8^{o}C (±0.07), and 38.9^{o}C (±0.07) for CTR, ECROF, and CROF respectively. No differences were found between treatment groups for calves` rectal temperature neither during treatment days (*P* = 0.90) nor after treatment (*P* = 0.94).

Table 3.1: Table regarding the effect of treatment on diarrhea, dehydration, calves` attitude (depression) and appetite (hypophagia), and oral electrolyte administration at pre-challenge, during treatment days (3 days consecutively), and after treatment cessation until 25 days of life. Pre-challenge data was collected once per calf, appetite and oral electrolyte administration measurements were not applicable. Control group was used as reference level for statistical comparisons. Data are presented as incidence (percentage of calves affected) and number of events (reported as mean and standard error of the mean).

		Pre-Challenge		During Treatment				After Treatment Cessation			
		Incidence	*P*	Incidence	*P*	Number of events	*P*	Incidence	*P*	Number of events	*P*
Diarrhea	CROF	0	1	84	0.44	1.9 ± 0.4	0.61	42	0.52	1.0 ± 0.5	0.2
	ECROF	0	1	58	0.74	1.3 ± 0.4	0.11	16	0.01	0.3 ± 0.5	0.02
	CTR	0	Ref.	68	Ref.	2.2 ± 0.4	Ref.	58	Ref.	1.9 ± 0.5	Ref.
Dehydration	CROF	30	0.72	84	0.79	2.2 ± 0.4	0.77	47	0.74	1.0 ± 0.6	0.14
	ECROF	33	0.73	58	0.29	1.4 ± 0.4	0.21	42	0.75	1.0 ± 0.6	0.14
	CTR	37	Ref.	79	Ref.	2.0 ± 0.4	Ref.	53	Ref.	2.3 ± 0.6	Ref.
Depressed	CROF	37	0.85	84	0.01	1.5 ± 0.3	0.09	53	0.19	1.4 ± 0.5	0.88
	ECROF	35	0.84	37	1	0.6 ± 0.3	0.69	37	0.95	1.0 ± 0.5	0.76
	CTR	32	Ref.	37	Ref.	0.8 ± 0.3	Ref.	32	Ref.	1.3 ± 0.5	Ref.
Hypophagia	CROF	*NA*		74	0.18	1.3 ± 0.3	0.73	42	0.51	1.2 ± 0.5	0.52
	ECROF	*NA*		47	0.74	0.9 ± 0.3	0.57	37	0.33	1.2 ± 0.5	0.47
	CTR	*NA*		53	Ref.	1.2 ± 0.3	Ref.	53	Ref.	1.7 ± 0.5	Ref.
Oral Electrolytes	CROF	*NA*		95	0.71	3.4 ± 0.5	0.51	58	0.50	1.8 ± 0.6	0.31
	ECROF	*NA*		74	0.70	1.9 ± 0.5	0.10	37	0.05	1.0 ± 0.6	0.06
	CTR	*NA*		84	Ref.	2.9 ± 0.5	Ref.	68	Ref.	2.7 ± 0.6	Ref.

CTR, Control group
CROF, Non-enteric coated crofelemer group
ECROF, Enteric-coated crofelemer group

Calves birth weight (kg) was not significantly different between treatment groups; 42.7 (±1.13), 42.4 (±1.13), and 40.2 (±1.10) for CTR, ECROF, and CROF, respectively (*P = 0.14*). At 10 days of life a statistical tendency was observed, average daily gain was; 177.4g/day (±43.9), 285.4 (±41.7), and 223.5 (±43.9) for CTR, ECROF, and CROF groups (*P = 0.08*, **Table 3.2**). No statistical difference was observed between treatment groups at 15 days of life, average daily gain was; 233.9 (±47.9), 292.9 (±42.9), and 254.0 (±46.5) for CTR, ECROF, and CROF groups (*P = 0.34*). At 25 days of life, average daily gain was; 219.2 (±47.9), 281.2 (±44.1), and 257.4 (±46.5) for CTR, ECROF, and CROF groups (*P = 0.43*).

Table 3.2: Table regarding calves birth weight and the effect of treatment on average daily weight gain at 10, 15, and 25 days of life. Data are presented as means and standard error of the mean.

	Treatment Groups			*P*-value
	CTR	ECROF	CROF	
Birth weight, kg	42.7 (1.13)	42.4 (1.13)	40.2 (1.10)	0.14
10 days ADG	177.4 (43.9)	285.4 (41.7)	223.5 (43.9)	0.08
15 days ADG	233.9 (47.9)	292.9 (42.9)	254.0 (46.5)	0.34
25 days ADG	219.2 (47.9)	281.2 (44.1)	257.4 (46.5)	0.43

CTR, Control group
ECROF, Enteric-coated Crofelemer group
CROF, Non-enteric coated Crofelemer group
ADG, Average daily weight gain calculated at each time point (g/day)

DISCUSSION

In the present study, two formulations of crofelemer extract were evaluated; an enteric-coated (ECROF) and a non-coated formulation (CROF). The ECROF formulation was significantly more effective in increasing fecal dry matter during treatment days when compared to CROF and placebo. Crofelemer has been studied for its antisecretory properties; crofelemer molecule can inhibit active chloride channels of enterocytes (Tradtrantip et al., 2010). Crofelemer targets the enterocyte extracellular surface and as such, an additional challenge is faced since the substance is susceptible to be washed away once in the lumen of the intestine due secreted fluid (Thiagarajah and Verkman, 2003). Tradtrantip et al., (2010) reported in vitro results indicating that crofelemer resisted washout, with less than 50% reversal of CFTR inhibition after 4 hours.

It is important to highlight that the effect reported by our study, favoring the enteric-coated form and not the non-enteric coated could be attributed to the enteric-coating process during the tablet formulation. Constable et al. (2005) reported that average abomasal pH was 3.22 during a 24 hours interval on calves fed all milk-protein milk replacer twice daily, and that the minimum preprandial pH was 1.34 and maximum postprandial pH was 6.07. The protection from the acidity of the abomasum by the enteric-coating process could have led to a higher concentration of the active component of the treatment into the intestinal lumen.

Fecal scores were used to visually assess calves affected with diarrhea. No differences were observed between treatment groups for the period of treatment. However, after treatment cessation, calves in the enteric-coated group had lower

incidence and lower number of diarrhea events. This finding is consistent with the data regarding oral electrolyte. Calves in the enteric-coated group had a tendency to received lower oral fluid therapy during treatment days and after treatment cessation.

In the current study, secretory diarrhea was induced by ETEC, which can rapidly lead to a high state of dehydration and life threatening conditions for newborn animals. As described by Hartmann et al. (1984, 1995) calves with watery diarrhea can lose up to 21% of its body weight. However, in the present study, no differences were found between treatment groups when evaluating dehydration during days of treatment. Nevertheless, diarrheic calves with suckling reflexes and moderate dehydration were given oral fluid therapy. It could be possible that the quick detection of dehydration and subsequent fluid therapy (oral electrolyte administration) prevented the induced secretory diarrhea from causing severe dehydration.

Serum total solids (TS) were used as a laboratory measurement of dehydration. In cases of secretory diarrhea, water moves from the circulatory system into the intestinal lumen, concentrating the solid components of blood, leading to a higher total solids concentration and higher reading by the optical refractometer. In the present study, control calves had a strong statistical tendency to have higher total solids readings during second day of treatment when compared to enteric-coated formulation of crofelemer extract treated calves. Moreover, after treatment cessation, control calves had higher serum total solids reading when compared to crofelemer treated calves. Laboratory evaluation of blood components can be used to measure dehydration status by the analyses of total solids, packed cell volume, creatinine, and urea. However, considerable variation on blood components could be observed when

supportive therapy is in place (Abutarbush and Petrie, 2007). Constable et al. (1998) reported that these parameters are not as good parameters to determine dehydration as degree of enophthalmos and total protein measurements.

All calves enrolled in this trial had failure of passive transfer. This was expected given the intentional reduced amount of colostrum replacer administered to the calves during the challenge. In our study, septicemia was expected in some calves due to the nature of the challenge; calves were given a high amount of bacteria with a low amount of colostrum. Neonatal calves are highly susceptible to septicemia because they are dependent on colostral immunoglobulins (Fecteau et al., 2009). However, we experienced a lower mortality rate, only one calf in each group was euthanized due septicemia.

Hyperthermia and hypothermia were not found to be significantly different between treatment groups in this trial at pre-challenge, during treatment days, and after treatment cessation (data not shown). Lower temperatures were found elsewhere to be significantly associated with hypovolemic shock in cases of severe dehydration (Sunderland et al., 2003; Nagy and Fekete, 2005).

Average daily gain was not significantly different between treatment groups. Calves on ECROF group had a statistical tendency to gain 108 grams per day more when compared to control group during the first 10 days of life. Newborn calves are more susceptible to fluid losses due to their higher total water content and higher extracellular fluid volume (Hartmann et al., 1984; Hartmann and Reder, 1995). It is possible that the effect observed on weight gain difference was due to water losses in the feces. A study conducted in the early 70s comparing non-diarrheic calves with

calves affected with spontaneous diarrhea reported that 96% of the weight lost in a diarrheic calf is due to water loss, in which 71.4%, 16.1%, and 12.5% of water loss was due fecal, urinary, and insensible, respectively (Phillips et.al., 1971). However, due the lack of calf starter consumption data little can be concluded from the data presented for the average daily weight gain calculated for the entire study period.

CONCLUSIONS

Newborn diarrhea was induced using ETEC. Diarrheic calves were treated with two forms of crofelemer extract (enteric and non-enteric coated) administered twice a day for 3 days consecutively and compared to control calves (placebo). Results herein presented demonstrated that enteric-coated crofelemer extract, a natural-product with anti-secretory properties can significantly increase fecal dry matter on diarrheic neonatal calves. More research is needed to test the efficacy of enteric coated crofelemer on incidence and severity of secretory diarrhea on diarrheic calves under natural challenge conditions.

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CHAPTER 4

PROPHYLACTIC USE OF SB-300 FOR THE PREVENTION OF NATURALLY OCCURRING DIARRHEA ON NEWBORN HOLSTEIN CALVES

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ABSTRACT

The objectives of this study were to evaluate the prophylactic use of SB-300, a standardized botanical extract on reducing fecal water losses and diarrhea events in Holstein bull calves individually housed under restricted whole milk feeding regime (6L/day) from 1 to 25 days of life. Furthermore, fluid therapy administration due to dehydration, weight gain, and fecal microbiome was also evaluated. Bull calves used in this study were born from normal parturition, fed 4L of pasteurized colostrum by esophageal feeder, and moved to a research facility at Cornell University. A double-blinded randomized clinical trial was designed to allocate a total of 40 newborn calves into one of two treatment groups: calves receiving twice daily a solution containing 500mg of SB-300 added to the whole milk for the first 15 days of life (SB-300, n = 20) or control group receiving sterile water added to the whole milk for the same period (CTR, n = 20). Treatment solutions had a total volume of 10mL per treatment. Data regarding fecal dry matter was collected to precisely measure water content in fecal samples and also to define diarrhea events; SB-300 group had significantly increased fecal dry matter during the study period. Additionally, significantly fewer diarrhea events were observed for calves in the SB-300 (16.9%) when compared to calves in the CTR group (46.5%). Dehydration status was evaluated and treated accordingly; calves with moderate dehydration were offered oral electrolyte and calves with severe dehydration were submitted to intravenous fluid therapy. Calves in SB-300 group had fewer intravenous fluid therapy administered during the study period (1.6%) when compared to CTR treatment group (3.1%). Overall fluid therapy

administered (oral electrolyte plus intravenous fluid) was significantly higher for CTR group (9.2%) when compared to SB-300 group (6.1%) during the study period. No differences in milk consumption, calf starter intake, weight gain were observed between treatment groups. A single time increase in *Bifidobacterium* was observed at 20 days of life for SB-300 group, no differences in fecal microbiome profile were reported between treatment groups. These results suggest that 500 mg of SB-300 added to the milk for 15 days can reduce incidence of diarrhea and reduce severe dehydration in milk fed calves.

INTRODUCTION

Neonatal calf diarrhea is a multifactorial disease that can be caused by infectious and non-infectious factors (Walker et al., 1998; O'Handley et al., 1999). In a report from the National Animal Health Monitoring System 2010, diarrhea was the most common disorder affecting pre-weaned heifers, with a nationwide incidence of almost 19% and the leading cause of death in pre-weaned heifers (NAHMS, 2011). Enteropathogens such as viruses, bacterial, and protozoa are often identified as etiological agents in calf diarrhea (Cho and Yoon, 2013).

Depending on the pathogen, calf diarrhea could have different pathophysiological mechanisms. These mechanisms have been well explained for *Escherichia coli* enterotoxin-mediated secretory diarrhea (Thiagarajah and Verkman, 2013), enterocytes villous atrophy-mediated malabsorptive diarrhea caused by *Cryptosporidium parvum* (Heine et al., 1984) and coronavirus (Lewis and Phillips, 1978), and enterocyte villus atrophy and chloride secretion-mediated malabsorptive and secretory diarrhea caused by rotavirus (Thiagarajah and Verkman, 2003). Briefly, the mechanism of which loose stools are produced could be divided in: secretory, malabsorptive, or both.

Extensive review on prevention and treatment of neonatal calf diarrhea is available (Constable, 2009). Fluid therapy is still the hallmark treatment for undifferentiated naturally occurring neonatal calf diarrhea. However, approximately 55% of calf operations facilities in the US make use of medicated milk replacers in attempt to control neonatal calf diarrhea and about 54% treat their scouring calves with antibiotics (Walker et al., 2012). The U.S. Food and Drug Administration

recommend five antimicrobials that can be used in medicated milk replacer for the control of bacteria, however; a maximum effort should be made to avoid routine administration of antibiotics as antimicrobial resistance could be a consequence of antimicrobial use (van den Bogaard and Stobberingh, 2000). Therefore, alternatives to antimicrobial for control and prevention of neonatal calf diarrhea are needed.

Recently, natural products with anti-secretory properties, proanthocyanidin oligomers, which are polyphenolic molecules extracted from the bark latex of the plant species *Croton lecheri*, had proved efficacy in reducing water losses measured in the fecal samples of neonatal Holstein bull calves were experimentally challenged with enterotoxigenic *E. coli* (Teixeira et al., 2015). This botanical extract has been studied for its antisecretory actions that involve the inhibition of two distinct chloride channels on the luminal membrane of the intestine; cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channel (CaCC) (Fisher et al., 2004; Tradtrantip et al., 2010).

Therefore, the objectives of this study were to evaluate the efficacy of daily doses of a standardized botanical extract with anti-secretory properties in Holstein calves with naturally occurring diarrhea by reducing intestinal water secretion, reducing dehydration and fluid therapy use, and reducing the incidence of diarrhea during the first 25 days of life. As a second objective, we investigated the effect of the anti-secretory extract on the intestinal microbiota.

MATERIALS AND METHODS

Experimental design, animals, and facility

This experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Cornell University (Protocol number 2013-0075). Sample size was calculated based on a fecal dry matter difference previously evaluated by a pilot study. Based on an expected mean difference of fecal dry matter between treatment groups of 1.4, a treatment group standard deviation of 1.2 and a control group standard deviation of 1.7, with a treatment group ratio of 1, assuming a type I error rate of 5%, a power of 80%, a sample size of 18 calves per group was calculated. As 10% of mortality was anticipated, a total of 20 calves per group were enrolled in this study.

The study design was a double-blinded randomized clinical trial. Randomization was performed a priori to beginning of the trial using Excel random function to create a balanced number of calves per treatment group (Microsoft). A total of 40 Holstein bull calves from one commercial dairy farm (Scipio Center, New York) were enrolled in the study. Newborn Holstein bull calves were monitored during parturition by on farm staff. Calves were eligible to be enrolled in the study if no assistance was required during parturition, no twins, and no birth defects detected by physical exam after parturition. To mimic farm conditions, 4 L of pasteurized (60^{o}C for 60min; T-300 GoodNature Products Inc.) pooled colostrum with Brix reading averaging 22.8% (ranging from 20.6% to 24.8%) was administered by esophageal tube to all calves within 45 minutes of birth (Oral Calf Feeder Bag with Probe, Jorvet).

All the calves were transported from the farm to the study site within 4 hours after birth. Briefly, calves were transported using an adapted vehicle for animal transportation; calves were kept inside individual crates with proper ventilation. Cleaning and sanitization of the vehicle was performed before and immediately after transportation.

The study site was a research barn with controlled temperature (20.6^{o}C, ranging from 19.4^{o}C to 21.8^{o}C) and humidity (50%, ranging from 45% to 55%) containing 20 individual stalls (2.1 square meters each) isolated by concrete walls, where calves were unable to have any contact with other calves and/or outside areas. A three step cleaning procedure was used for water buckets, feed buckets, and bottles after each feeding; consisting of rinsing all the equipment with lukewarm water, scrubbing a mixture of hot water and alkaline detergent solution, and finally rinsing in chlorinated water. The research barn used to conduct this trial was used twice; since limited space was available (20 stalls). A first run was performed from January to March of 2016 and the second run from March to May of 2016. The two runs were performed using the same exact procedures for enrollment, data collection, cleaning, and laboratory procedures.

Calves were fed saleable whole milk purchased from the Cornell Teaching Dairy (Cornell University) twice daily (0600h and 1800h) from first feeding to the end of study period at 25 d of life. From day one, calves were gradually encouraged to drink from the bucket. Water was available ad-libitum from day 1 until the end of the study. All calves were offered calf starter starting of day 16 of life (Calf starter 18% CP, DuMOR).

Treatment Administration and Data Collection

Randomization was performed in blocks (by dam parity and birth weight) a priori using random function in Excel (Microsoft). Calves were randomly assigned into 1 of 2 treatment groups; control (CTR; $n = 20$) or standardized botanical extract (SB-300; $n = 20$).

Treatment consisted of 500mg of SB-300 diluted in 10mL sterile water (VetOne) using a conical twisted top sterile tube (Corning Falcon). Treatments were prepared by adding 10mL of sterile water and 500mg of SB-300 into the 15ml conical tube and placing them in a benchtop tube mixer at low speed for 1 hour before each feeding (TubeRevolver, SoCal Biomedical). Treatments were administered twice daily during feeding by adding the solution into the milk for the first 15 days of life. Control calves were administered 10mL of sterile water by adding the solution into the milk, twice daily, for the first 15 days of life.

To accomplish double-blindness, one member of the research team only performed randomization and calf stall assignment. Another member of the research worked full time and was responsible for morning and evening feedings and treatments; treatments were added to the milk by the time of feeding. A third member of the research team was responsible for collecting the data.

Calves were weighed at birth, 5, 10, 15, 20, and 25 days of life using a digital portable scale (Waypig-15, Vittetoe Inc.). Rectal fecal swabs for metagenomic data were also collected at birth, 5, 10, 15, 20, and 25 days of life using DNA-free sterile swabs (6” Sterile DNA-Free Cotton Swab, Puritan Medical Products Company LLC) , placed into a 1.5ml sterile conical flask, number identified, and placed in a -80^{o}C

freezer within 10 min of sampling. Blood samples were collected via jugular venipuncture using an 18-gauge by 3.8cm needle into 10mL vacuum tubes (Becton, Dickinson and Co.). Blood sampling was performed at the second day of life. Serum was harvested following centrifugation at 2,000 × *g* for 15 minutes at 4°C. Serum IgG was measured using the second blood collection (2^{nd} day of life) using a radial immunodiffusion assay according to kit instructions (Bethyl Laboratories Inc.).

All the calves were examined twice daily, within 2 hours after feeding from day 1 until day 25 of life. The following parameters were evaluated during each examination: rectal temperature (Digital Rectal Thermometer), eyes recession into the orbit, skin tent, attitude (suckling reflexes, standing position, and resting position), milk consumption, and calf starter intake (from 15 to 25 days of life).

Additionally, fecal samples were collected via digital rectal stimulation, sample was collected twice daily within one hour after feeding onto aluminum weighing boats from day 1 to day 15, at day 20, and a last collection performed once at 25 days of life and immediately used for dry matter evaluation. For all fecal samples collected, determination of fecal dry matter was performed as described by Bellosa et al., (2011). Briefly, samples were weighed on a precision digital scale (6202-1S, Sartorius) and then dried at 108^{o}C for 24 hours (Model 10 Lab Oven, Quincy Lab) and re-weighed immediately to determine the percent dry matter.

A fixed amount of saleable whole milk was offered for the entire study period (3 L/feeding; 6 L total per day); milk consumption was measured after each meal from day one to the last day of the study. Calf starter was only offered after treatment cessation (treatment ceased on day 15 after second feeding); 4 kg of calf starter was

offered and weighed again at 25 days of life, average calf starter intake was calculated from day 16 until day 25 by subtracting the calf starter offered final weight (day 25) by the initial weight (day 16) and dividing by the 9 days.

Dehydration was assessed twice daily and fluid therapy was administered accordingly. Briefly, calves with bright eyes and skin feels pliable of a slight loss of skin elasticity (< 3 seconds), and eyes not recessed into orbit (≤ 2 mm) were considered not dehydrated and no rescue was performed. Calves with skin tent > 3 seconds but < 10 seconds, eyes slightly recessed into orbit (>2 mm but ≤ 4 mm) and with sucking reflexes were considered moderate dehydrated and given oral fluid therapy. Oral hydration was also performed with a commercial oral electrolyte (Hydralyte, Lloyd Inc.); oral electrolyte was fed via bottle as an extra meal administered at time of physical exam (2 hours after each feeding). Calves with severe dehydration (skin tent > 10 seconds), eyes markedly recessed into orbit (≥ 5 mm), with or without suckling reflexes were considered severely dehydrated and given intravenous rescue therapy. Intravenous fluid therapy using isotonic sodium bicarbonate was administered by jugular vein catheter (70 mL/kg/hour).

Next Generation Sequencing Methodology

Isolation of DNA from fecal content was performed by adding the cotton head of each sample to 1.5 ml of nuclease-free water (Life Technologies), and vortexed for two minutes. The swab was then removed and the sample was centrifuged for 10 minutes at 13,200 x g. The supernatant was discarded and the remaining pellet was used for total metagenomic DNA extraction using the E.Z.N.A Stool DNA Kit

(Omega Bio-Tek) following manufacturers guidelines. Concentration and purity of the DNA were evaluated by optical density using a spectrophotometer (NanoDrop Technologies) and approximately 350ng of DNA was used for PCR procedure.

The 16S rRNA gene was then amplified by PCR from individual metagenomic DNA samples from the fecal content using barcoded primers. For amplification of the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene, primers 515F and 806R were used according to previously described methods and optimized for the Illumina MiSeq platform (Caporaso et al., 2012). The earth microbiome project (hearthmicrobiome.org) was used to select 280 different 12 base pair barcodes for the 16S rRNA PCR, as previously described (Caporaso et al., 2012). The 5'-barcoded amplicons were generated in triplicate using 300 ng of template DNA, a master mix (Lucigen), and 10 µM of each primer. The PCR conditions for the 16S rRNA gene included an initial denaturing step of 94°C for 3 minutes followed by 35 cycles of 94°C for 45 seconds, 50°C for 1 minutes and 72°C for 90 seconds and a final elongation step of 72°C for 10 minutes. Replicate amplicons were pooled and purified with a QIAquick PCR Purification Kit (Qiagen) and visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/ml ethidium bromide before sequencing.

Statistical Analyses

Descriptive statistical analysis was undertaken in SAS (SAS Institute Inc.). An ANOVA was used to evaluate differences in birth weight (kg) at enrollment, serum immunoglobulin (IgG, g/L) collected at 2 d of life, and baseline fecal dry matter (%).

Additionally, the intraassay and interassay coefficients of variation for serum IgG quantified by an ELISA kit were 3.0 and 3.8%, respectively. Only 2 calves in the SB-300 group and 1 calf in the control group died. Data from these calves were excluded from all statistical analysis. (2 days into the study). Differences in mortality was evaluated by Fisher`s exact test using the FREQ procedure in SAS. Briefly, all three calves were presenting persistent fever (rectal temperature > 39.5^{o}C) from first feeding, depression (lost appetite, recumbency). Calves were defined as septicemic and antibiotic therapy with a supportive dose of corticosteroids and intravenous fluid administration was used, upon unsuccessful recover, euthanasia was performed.

To evaluate the effect of treatment on daily milk intake, daily calf starter intake, and daily weight gain, five general linear models were fitted to the data using the GLM procedure of SAS. The independent variables offered to the models were; treatment group (CTR and SB-300), serum Immunoglobulin-G at 2 days of life (g/L), body weight at enrollment (kg), baseline fecal dry matter (%). Treatment group was the only variable forced into the models and a backward stepwise variable selection was performed. The assumption that the residuals were normally distributed was assessed by visually evaluating the distribution plot of the Studentized residuals.

To evaluate the effect of treatment on fluid therapy (oral electrolyte, intravenous fluid, and overall fluid therapy), three multivariable mixed logistic regression models were fitted to the data using the GLIMMIX procedure of SAS. The independent variables offered to the model were; treatment group (CTR and SB-300), serum Immunoglobulin-G (g/L), body weight at enrollment (kg), and baseline fecal dry matter (%). Odds ratio and adjusted probabilities of diarrhea were obtained using

the LSMEANS statement.

A mixed general linear mixed model was fitted to the data using the MIXED procedure of SAS to analyze the effect of treatment on fecal dry matter (%).The independent variables offered to the model were; treatment group (CTR and SB-300), serum Immunoglobulin-G (g/L), body weight at enrollment (kg), and baseline fecal dry matter (%). Fecal dry matter data were longitudinally collected and consisted of a total of 33 measurements per calf: twice daily from day 1 to 15, twice daily at day 20, and one sample collected at 25 days of life before the end of the study. Therefore, data points were correlated within each research subject. To account appropriately for within-calf correlation, the error term was modeled by imposing a first-order autoregressive covariance structure. For this model a backward stepwise variable selection was performed. Furthermore, to assess the effect of treatment on fecal dry matter collected during the study period, the interaction between treatment and days was forced into the model. Visual evaluation of the distribution plot of the Studentized residuals allowed us to assume that the residuals were normally distributed.

To avoid subjectivity from visually scoring calves with diarrhea based on fecal consistency, diarrhea was defined after data on fecal dry matter were collected. The event of diarrhea was given to a calf that presented at least one of the twice daily measurements of fecal dry matter $\leq 10.0\%$. Data regarding events of diarrhea was created for each day from 1 to 15, at 20, and at 25 days of life. To evaluate the effect of treatment on diarrhea (yes or no), a multivariable mixed logistic regression model was fitted to the data using the GLIMMIX procedure of SAS. The independent variables offered to the model were; treatment group (CTR and SB-300), serum

Immunoglobulin-G (g/L), body weight at enrollment (kg), and baseline fecal dry matter (%). Adjusted probability of diarrhea was obtained using the LSMEANS statement.

The effect of treatment on body weight collected at 1, 5, 10, 15, 20, and 25 days of life was evaluated by a mixed general linear model using the MIXED procedure of SAS. To control for repeated measures of body weight, the animal identification number (nested within run) was included in all models as a random effect and the independent variables offered to the model were treatment group (CTR and SB-300), serum Immunoglobulin-G (g/L), body weight at enrollment (kg), and baseline fecal dry matter (%). For this model a backward stepwise variable selection was performed and to assess the effect of treatment on fecal dry matter collected during the study period, the interaction between treatment and days was forced into the model. The assumption that the residuals were normally distributed was assessed by visually assessing the Studentized residuals plot.

Abundance of the individual genera; Bifidobacterium, Lactobacillus, Faecalibacterium, and Escherichia were evaluated using 4 similar general linear mixed models in SAS. Variables offered to the models included treatment group (CTR and SB-300), days (1, 5, 10, 15, 20, and 25 days of life), and the interaction terms between these two variables. To account appropriately for within-calf correlation, the error term was modeled by imposing a first-order autoregressive covariance structure.

RESULTS

Forty newborn Holstein bull calves were enrolled in this study. No differences in mortality was observed, 2 calves in the SB-300 group and 1 calf in the CTR group ($P = 0.38$). Birth weight was also not different between CTR (37.6 kg, 95% Confidence Interval = 37.4 – 38.4) and SB-300 (37.7 kg, 95% CI = 37.4 – 38.4). Serum IgG quantification was performed at the second day of life and no differences were found between calves in the CTR group (12.3 g/L, 95% CI = 11.6 – 13.0) and SB-300 group (12.1 g/L, 95% CI = 11.4 – 12.8). Additionally, no differences were found for baseline fecal dry matter between treatment groups ($P = 0.55$, **Table 4.1**).

Table 4.1: Newborn Holstein bull calves were fed pasteurized pooled colostrum, individually housed, fed restricted whole milk (6L/day) from day 1 to 25 days of life, and offered calf starter from day 16 to 25 of life. Descriptive table regarding number of calves enrolled in each group, percentage of calves that died during the study period, serum immunoglobulin G concentration (IgG, g/L), birth weight (kg), and baseline fecal dry matter (%) is presented. Results are presented as means and respective 95% confidence interval (95% CI).

	Control		SB-300		*P*-value
n	20		20		
Mortality, %	5%		10%		0.38
	Means (95% CI)				
Birth weight, kg	37.6	(37.3 - 37.9)	37.7	(37.4 - 38.4)	0.57
Serum IgG, g/L	12.3	(11.6 - 13.0)	12.1	(11.4 - 12.8)	0.72
Fecal dry matter baseline, %	29.8	(23.5 - 36.1)	27.2	(20.9 - 33.5)	0.55

SB-300, calves in the standardized botanical extract group

Milk Intake, Calf Starter Intake, and Average Daily Gain

No differences were observed for milk and calf starter intake between treatment groups (**Table 4.2**). Briefly, calves in the CTR group had an average milk intake of 5.38 L/d (95% CI = 5.11 – 5.65) and calves in the SB-300 group an intake of 5.55 L/d (95% CI = 5.28 – 5.83). Calf starter was only offered after treatment cessation at 16 d of life. Calf starter intake was not significantly different between treatment groups (P = 0.69). Moreover, no differences on average daily weight gain was observed from 1 to 25 days of life; calves in the CTR group had an average daily weight gain of 0.38 kg/d (95% CI = 0.27 – 0.49) and calves in the SB-300 group had an average of 0.43 kg/d (95% CI = 0.30 – 0.55).

Table 4.2: The effect of treatment on milk consumption from day 1 to 25 days of life, calf starter intake from day 16 to 25 days of life, and the effect of treatment on average daily gain (divided in 3 periods; 1 to 15 days, 15 to 25 days, and from 1 to 25 days of life) were evaluated by 5 different general linear mixed models. Results are presented as means and respective 95% confidence interval (95% CI).

n		Control 19	SB-300 18	*P*-value
	Period	Means (95% CI)		
Milk intake, L/day	1 to 25	5.38 (5.11 – 5.65)	5.55 (5.28 – 5.83)	0.29
Calf starter intake, kg/day	15 to 25	0.16 (0.15 – 0.17)	0.16 (0.15 – 0.17)	0.69
	1 to 15	0.14 (0.12 – 0.18)	0.17 (0.14 – 0.21)	0.22
Weight gain, kg/day	15 to 25	0.74 (0.56 – 0.92)	0.89 (0.71 – 1.08)	0.22
	1 to 25	0.38 (0.29 – 0.47)	0.46 (0.37 – 0.56)	0.22

SB-300, calves in the standardized botanical extract group

Dehydration and Fluid Therapy

During the study period, no differences were observed between treatment groups when evaluating the odds of a calf being moderately dehydrated and needing oral electrolytes (OR = 0.07, *P* = 0.09, **Table 4.3**). The odds of a calf being severely dehydrated and consequently needing intravenous fluids was 0.5 lower for calves in the SB-300 group when compared to calves in the CTR group (*P* = 0.04). Likewise, the odds of needing any fluid therapy (due moderate and severe dehydration) during the study period were 0.6 times lower in the SB-300 group when compared to the CTR group.

Table 4.3: Dehydration was assessed twice daily for all calves in the study from day 1 to 25 days of life; calves with moderate dehydration were offered oral electrolyte and calves with severe dehydration were rescued with intravenous fluid therapy. The effect of treatment on fluid therapy was evaluated by 3 generalized linear mixed models. Calves in the control group were used as a reference level. Adjusted probabilities, odds ratio and the respective 95% confidence interval (95% CI) are displayed.

	Adjusted Probability		OR (95%CI)	*P*-value
	Control	SB-300		
Oral Electrolytes	6.3%	4.5%	0.7 (0.5 - 1.0)	0.09
Intravenous Fluids	3.1%	1.6%	0.5 (0.3 - 0.9)	0.04
Overall Fluid Therapy	9.2%	6.1%	0.6 (0.4 - 0.9)	0.01

SB-300, calves in the standardized botanical extract group

Fecal Dry Matter and Diarrhea

A significantly higher fecal dry matter percentage was observed for calves in the SB-300 group (18.2 %, 95% CI = 17.2 – 19.2) when compared to calves in the CTR group (13.2 %, 95% CI = 12.2 – 14.2) during the study period (*P* <.0001, **Figure 3.1**). Likewise, when evaluating the effect of treatment on events of diarrhea, calves in

the SB-300 group had significantly fewer events of diarrhea (16.9 %, 95% CI = 12.4 – 22.5) when compared to calves in the CTR group (46.5 %, 95% CI = 39.9 – 53.2) during the study period (P <.0001, **Figure 4.2**). For both outcomes, the interaction term between treatment group and days of life was not significant and was forced in the models to generate daily estimates for both, fecal dry matter (P = 0.22) and diarrhea (P = 0.75).

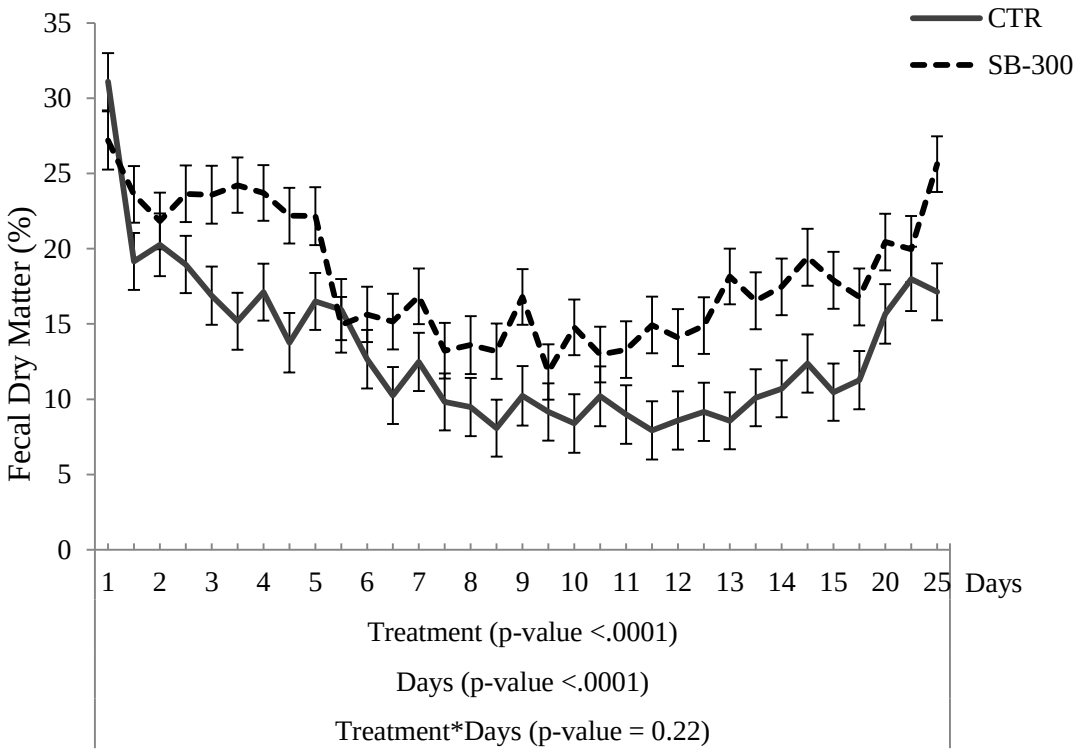

Figure 4.1: Fecal dry matter was performed twice daily for each of the treatment day (day 1 to 15). Additional samples were collected twice at day 20 and once at day 25 before the end of the study. Treatments were administered twice daily with whole milk for the first 15 days of life. The effect of treatment, days, and the interaction between treatment and days are also displayed. Solid line represents calves in the control group (CTR, n = 19) and dashed line represents calves in the standardized botanical extract group (SB-300, n = 18). Y axis represents fecal dry matter least square means and X axis represents days into the study. Errors bars are displaying standard errors.

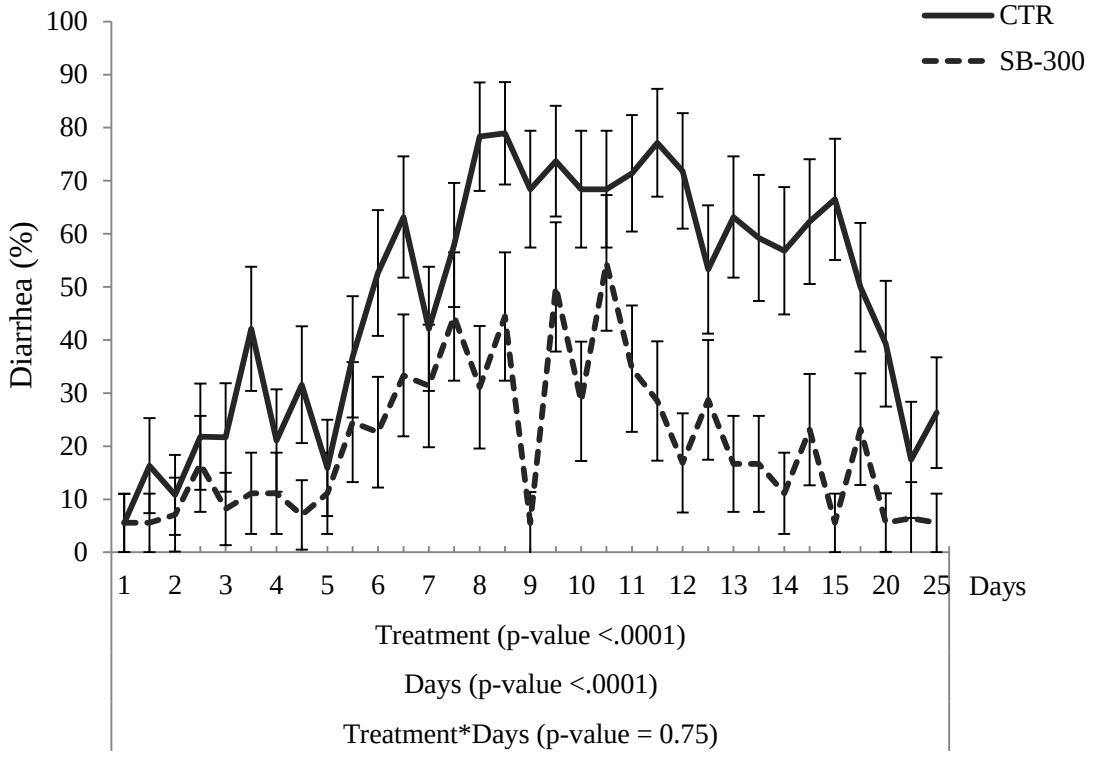

Figure 4.2: Diarrhea was recorded every day for all the calves in the study; an event of diarrhea was confirmed when a calf fecal sample presented at least one of the twice daily measurements of fecal dry matter equal or less than 10.0%. Treatments were administered twice daily with whole milk for the first 15 days of life. The effect of treatment, days, and the interaction between treatment and days are also displayed. Solid line represents calves in the control group (CTR, n = 19) and dashed line represents calves in the standardized botanical extract group (SB-300, n = 18). Y axis represents the model adjusted proportion of calves with diarrhea and X axis represents days into the study. Errors bars are displaying standard errors.

Body Weight

No effect of treatment was observed for body weight data collected during the study period (P = 0.42, **Figure 4.3**). The interaction term between treatment group and days of life was not significant and was forced into the model (P = 0.14). Briefly, body weight by the end of the study period (25 d of life) was 49.2 kg (95% CI = 47.7 – 50.6) for calves in the SB-300 group and 47.0 (95% CI = 45.6 – 48.5) for calves in the CTR group.

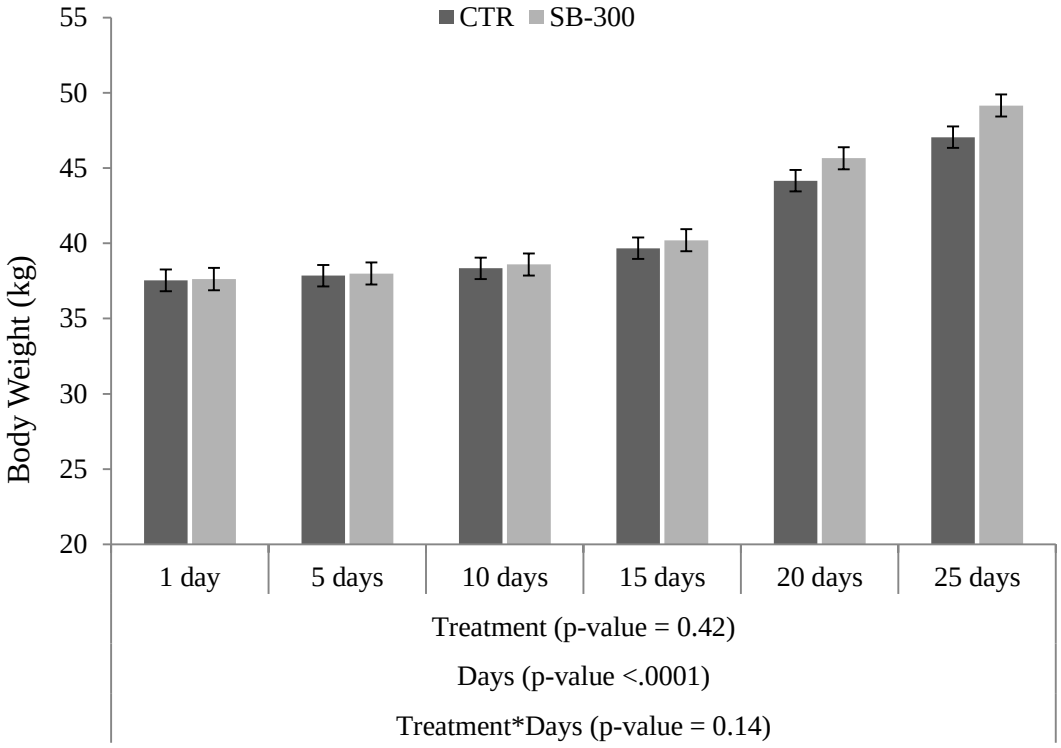

Figure 4.3: The effect of treatment on body weight collected at 5, 10, 15, 20 and 25 days of life was evaluated by a general linear mixed model. The effect of treatment, days, and the interaction between treatment and days are displayed. Y axis represents the least square means of body weight and the X axis represents the days of body weight measurement. Dark gray bars represent calves in the control group (CTR, n = 19) and light gray bars represent calves in the standardized botanical extract group (SB-300, n = 18). Standard errors are displayed as error bars.

Fecal Microbiome

The three most abundant phyla described in this study were *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* regardless of the treatment group. No differences in mean relative abundance of each phylum within days of life between treatment groups were observed (**Figure 4.4**). To investigate beneficial genera of intestinal bacteria, an individual evaluation of the following bacterial genera was performed; *Bifidobacterium* (Vlkova et al., 2006; Bunesova et al., 2012), *Lactobacillus* (Ewaschuk et al., 2004), and *Faecalibacterium* (Oikonomou et al., 2013; Foditsch et al., 2015). Additionally, the *Escherichia* genus was also considered for its known association with unhealthy microbiome (Acres, 1985). *Bifidobacterium* had a higher mean relative abundance at 20 d of life for SB-300 calves when compared to CTR calves ($P < 0.05$). However, that was the only difference observed from data regarding genera; no differences were found between any of the mean relative abundance of genera within days between treatment groups (**Figure 4.5**).

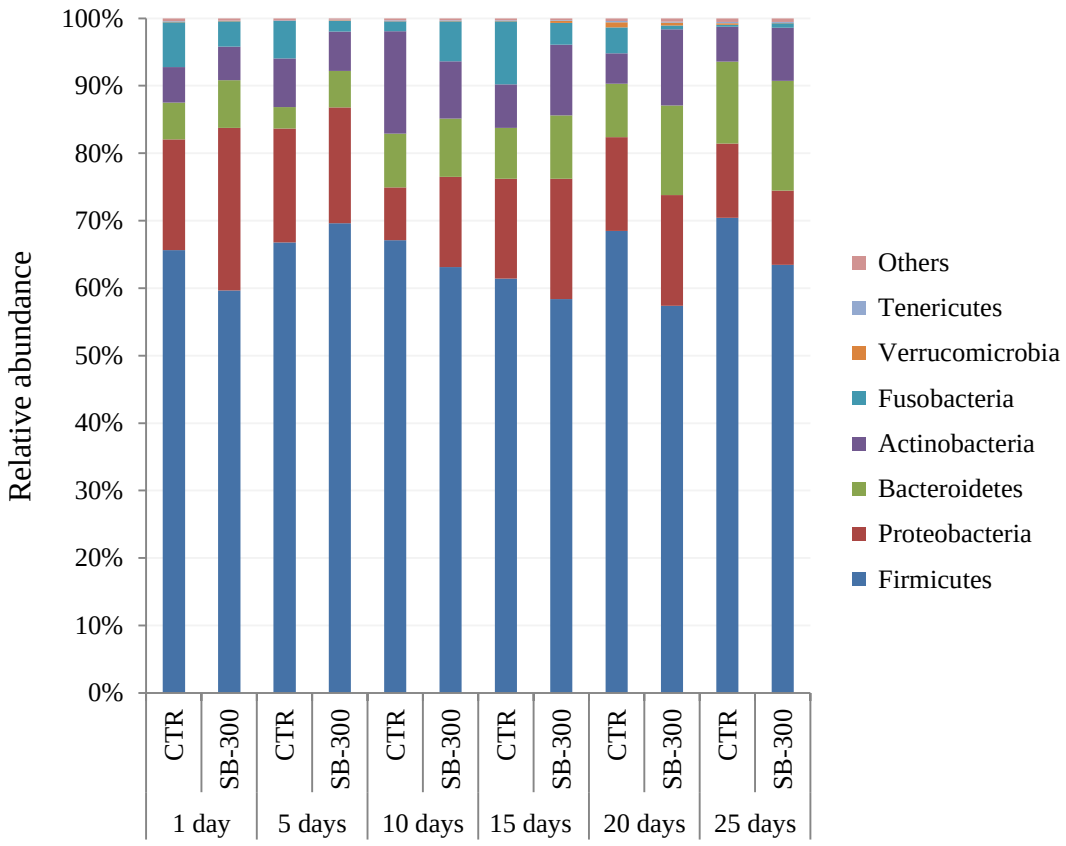

Figure 4.4: Descriptive distribution of the mean relative abundance of the most prevalent bacterial phyla identified in fecal samples of control calves (CTR, n = 19) and calves receiving standardized botanical extract (SB-300, n = 18). Fecal samples were collected once daily at 1, 5, 10, 15, 20, and 25 days of life.

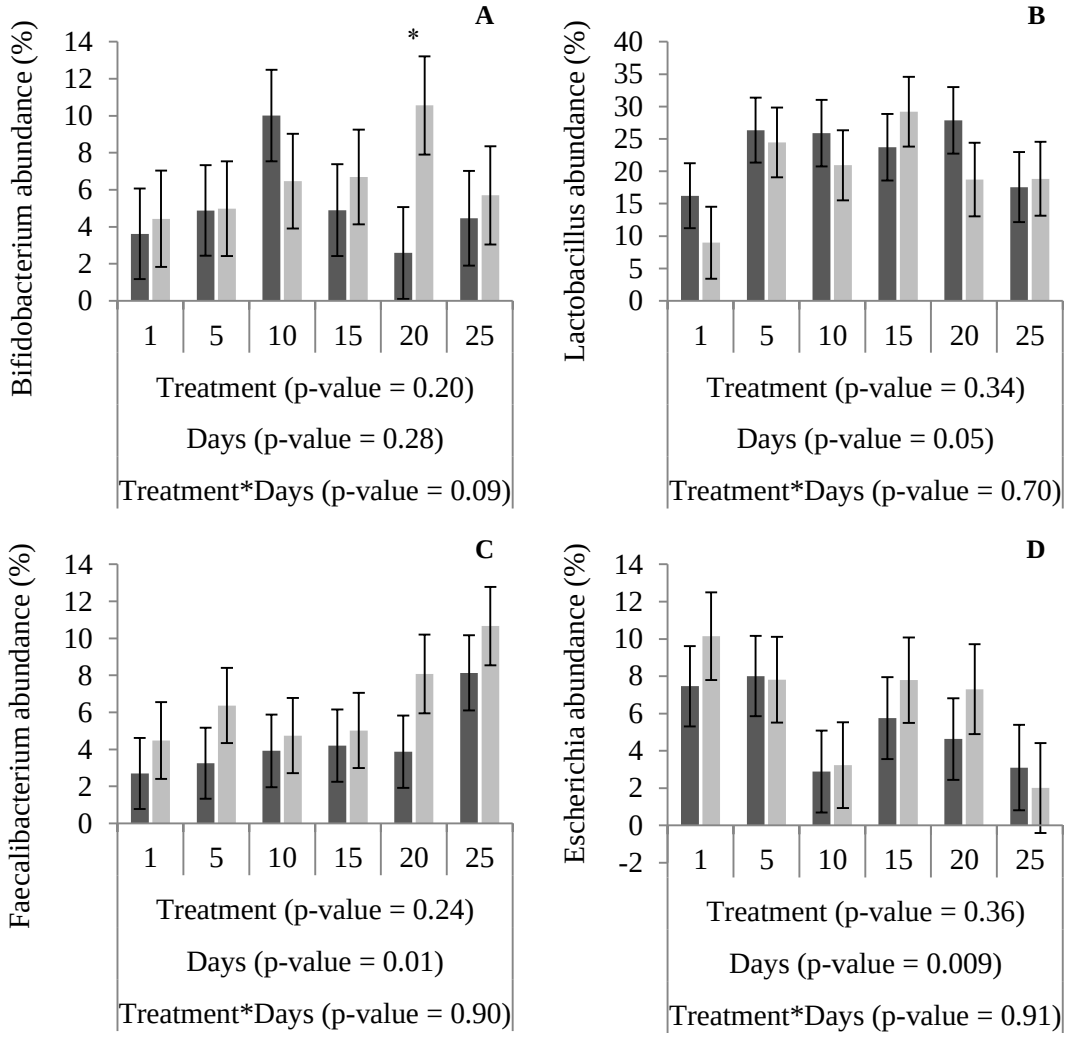

Figure 4.5: Comparison of mean relative abundance of three genera previously associated with healthy gut microbiome represented by *Bifidobacterium* (A), *Lactobacillus* (B), *Faecalibacterium* (C), and one genus related to unhealthy microbiota represented by *Escherichia* (D) found in fecal samples. Y axis represents the mean relative abundance in percentage and the X axis represents the data point of each fecal sample collected (days of sampling). Dark gray bars represent calves in the control group (CTR, n = 19) and light gray bars represent calves in the standardized botanical extract group (SB-300, n = 18). Standard errors are displayed as error bars. Statistical significance adjusted by Bonferroni within each day is presented as * ($P <$ 0.003).

DISCUSSION

This double-blinded randomized clinical trial evaluated the prophylactic use of a standardized botanical extract administered twice daily at feeding mixed with saleable whole milk for the first 15 days of life on naturally occurring diarrhea. Diarrhea was precisely defined by measuring dry matter content in fecal samples collected twice daily from day 1 to 15, at 20, and at 25 days of life. Decreased incidence of undifferentiated naturally occurring diarrhea (defined as fecal dry matter ≤ 10.0%) and fewer administration of fluid therapy (due to severe and moderate dehydration) in Holstein bull calves housed individually and fed restricted amount of saleable whole milk for 25 days were the main findings of this study. No differences were found in milk consumption, average daily weight gain. No effect of treatment on the intestinal microbiome was observed besides a transitory increase for the genera *Bifidobacterium* at 20 days of life for calves in the SB-300 groups when compared to control group calves.

Four main enteropathogens are associated with neonatal calf diarrhea; enterotoxigenic *Escherichia coli* as the main secretory-induced diarrhea pathogen, *Cryptosporidium parvum* and coronavirus are the main villus atrophy malabsorptive-induced, and rotavirus capable of causing diarrhea by both pathways (Reynolds et al., 1986; Varshney et al., 1995; Gulliksen et al., 2009b; Cho and Yoon, 2013). These pathogens can induce secretory diarrhea by increasing chloride secretion in the intestinal lumen, consequently leading to intestinal fluid hyper secretion (Thiagarajah and Verkman, 2003). Polyphenolic molecules isolated from the bark latex of *Croton lechleri* of the family *Euphorbiaceae*, have being extensively studied for its anti-

secretory properties (Fisher et al., 2004). These standardized botanical extracts are inhibitors of two distinct chloride channels which are responsible for intestinal chloride secretion; cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channel (CaCC) (Thiagarajah et al., 2004). Previously, the antisecretory properties of the standardized botanical extract in a single agent challenged secretory-induced diarrhea set-up, was proven to significantly decrease water content in fecal samples (Teixeira et al., 2015).

The antisecretory properties of the SB-300 are very specific therefore, the differences found in fecal dry matter percentage and consequently events of diarrhea can only be due its effect of blocking enterocytes chloride channels avoiding water sequestration into the intestinal lumen. In the current study, a twice daily dose of 500 mg per feeding (3L whole saleable milk) of SB-300 for the first 15 days of life was able to reduce water secretion in fecal samples and consequently reduce the incidence of diarrhea. Mixed infection are often reported in calves suffering from naturally occurring diarrhea (Reynolds et al., 1986). The diarrhea affecting calves in this trial can be considered undifferentiated naturally occurring diarrhea, since no attempt to identify the cause of diarrhea was made. The prevalence of pathogens capable of producing secretory-induced neonatal diarrhea was elsewhere described to range between 2.0% to 45.0% and 17.0% to 80.0% for enterotoxigenic *Escherichia coli* and rotavirus, respectively (Reynolds et al., 1986; Frank and Kaneene, 1993; Luginbuhl et al., 2005; Gulliksen et al., 2009a; Bartels et al., 2010; Smith, 2012; Cho et al., 2013; Klein-Jöbstl et al., 2014).

Assessing dehydration and accurately identifying a calf that requires fluid

therapy is very important. During an event of secretory diarrhea, a calf can lose significant amount of body fluids and with it blood electrolytes (Dalton et al., 1965; Phillips et al., 1971; Fisher and De la Fuente, 1972). Dehydration can be successfully treated and provide calves with the necessary electrolytes and nutrients to restore its hydration status (Lewis and Phillips, 1978; Groutides and Michell, 1990; Hartmann and Reder, 1995; Sen et al., 2009; Smith, 2009). As previously described in calves with induced dehydration and diarrhea, the best clinical predictor of dehydration in neonatal calves are the degree of enophthalmos and neck skin-tent duration (Constable et al., 1998).

In the current trial, degree of dehydration was assessed by both described methods and fluid therapy was performed accordingly. Oral electrolyte administration was provided when a calf was found with moderate dehydration; no differences were found between treatment groups. However, a significantly higher number of intravenous fluids due to severe dehydration were administered for calves in the control group when compared to calves in the SB-300 group. Finally, overall fluid therapy administration (oral and intravenous fluids) was significantly different between treatment groups. In this study, calves suffering from undifferentiated diarrhea could have benefited from the constant dose of the standardized botanic extract offered twice daily for 15 days; as reported by Phillips et al, (1971) neonatal calves suffering from secretory diarrhea can lose up to 71.4% of water in its feces and reducing this intestinal water secretion can reduce the severity of dehydration.

In this present study, calves were offered a restricted amount of milk per day (6L) from day 1 to 25 days of life and calf starter was only offered after treatment

starting at day 16 until 25 d of life. Milk was offered even if a calf was under fluid therapy, as reported by others, calves suffering from diarrhea would benefit from the energy provided by the milk offered during feeding (Heath et al., 1989; Garthwaite et al., 1994). No differences were found between daily milk consumption and starter intake, in addition to no effects on average weight gain. The standardized botanical extract is a non-absorptive compound that exerts its inhibition function from the extracellular side of the enterocytes, having no effect on the enterocyte function as nutrient absorption (Holodniy et. al., 1999). In a multi-herd study evaluating risk factors that could impair calf growth, neonatal calf diarrhea was one of the risk factors associated with lower body weight gain (Virtala et al., 1996; Windeyer et al., 2014). In the current study, besides the higher incidence of diarrhea observed for calves in the control group, body weight was not significantly different between treatment groups during the study period.

Here, we investigate the effect of a botanical extract on the fecal microbiota using a metagenomic approach. In this study, the hypothesis behind the microbiome investigation was that; a lower secretion of water during an event of secretory diarrhea would lead to a more stable microbiome, possibly favoring beneficial bacteria that could lead to an improved performance. During events of diarrhea triggered by *E. coli*, rotavirus, and coronavirus the intestinal lumen can create a favorable environment for pathogenic bacteria overgrowth (Tzipori et al., 1981; Tzipori et al., 1983; Kaske, 1993). The neonatal calf intestinal microbiota is dynamic during the first week of life and different intestinal microbiota profiles can be associated with disease and weight gain (Uyeno et al., 2010; Oikonomou et al., 2013; Malmuthuge et al., 2015). In

neonatal calves, the beneficial effects of decreasing diarrhea incidence and improving weight gain in calves have been reported when a higher prevalence of certain bacterial genera are provided, namely *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* (Abe et al., 1995; Foditsch et al., 2015). In the current study, fecal swabs were collected during treatment days for metagenomic sequencing and no differences in relative abundance between treatment groups were detected for genera *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium*. Fecal swabs were also collected at day 20 and at day 25 of life; a difference was observed at day 20 only for *Bifidobacterium* between treatment groups, where calves in the SB-300 group had a higher relative abundance of *Bifidobacterium* when compared to calves in the control group. However, by day 25 no differences were reported.

CONCLUSIONS

Forty newborn Holstein bull calves fed pasteurized pooled colostrum were raised under restricted feeding of saleable whole milk, housed individually from day 1 to 25 days of life. A prophylactic dose of 500mg of SB-300 was added to the milk twice daily at feeding times for 15 days and diarrhea events were precisely identified by percentage of water in the feces. Calves in the SB-300 treatment group experienced significantly fewer events of diarrhea during the study period (16.9%) when compared to CTR calves (46.5%). Calves in the CTR group suffered more from severe dehydration and had higher intravenous fluid therapy intervention. No effect of treatment on average milk intake, calf starter intake, and average daily weight gain was observed. Besides a single time increase in *Bifidobacterium* observed at day 20

for SB-300 group, no differences in fecal microbiome profile was reported between treatment groups. Combined, these results suggest that 500 mg of SB-300 added to the milk during feeding for 15 days can reduce incidence of diarrhea and reduce severe dehydration in milk fed calves.

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CHAPTER 5

EFFICACY OF TILDIPIROSIN METAPHYLAXIS ON THE PREVENTION OF RESPIRATORY DISEASE, OTITIS, AND MORTALITY IN PRE-WEANED HOLSTEIN CALVES

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ABSTRACT

The objective of this study was to evaluate the efficacy of two metaphylaxis approaches (long acting antibiotic injected once at 10 days of life or twice at 10 and 35 days of life) on the prevention of bovine respiratory disease (BRD), otitis, and mortality in high-risk group-housed pre-weaned Holstein heifer calves. The antibiotic of choice for the metaphylactic approach was a long acting macrolide (tildipirosin) administered subcutaneous on the base of the neck at a dose of 1ml for 45 kg of body weight. A clinical trial was carried out on one dairy farm with random allocation of newborn calves to one of 3 treatments: 1) control (CRT), 2) one injection at 10 days of life (M1), and 3) two injections at 10 and 35 days of life (M2). Study heifers (n = 795) were reared in group pens of 25 calves per pen and fed unrestricted acidified non-saleable milk from day 1 until 65 days of life. Cox proportional hazard and general linear mixed models were used to evaluate the effect of treatment on mortality, BRD and otitis, and average daily weight gain.

Proportion of calves with inadequate transfer of passive immunity, birth weight, proportion of calves born from primiparous dams, and proportion of calves born from assisted parturitions were not different between CTR, M1, and M2 treatments, respectively. A statistical tendency to have lower hazard of BRD was observed for M1 (HR = 0.68, P = 0.07) when compared to CTR group. Calves in M2 group also had a tendency for a lower hazard of BRD when compared to CTR group (HR = 0.70, P = 0.09).

Additionally, a significantly lower hazard of having the combined BRD and otitis events was observed for M1 (HR = 0.70, P = 0.009) and M2 (HR = 0.72, P =

0.01). Metaphylactic treatments had no effect on mortality, otitis, and average daily weight gain during the pre-weaning period.

INTRODUCTION

The National Animal Health Monitoring Survey (NAHMS) reported that bovine respiratory disease (BRD) accounts for 22.5% of all pre-weaned dairy heifer mortality in the United States (USDA, 2007). Bovine respiratory disease is a multifactorial process which is typically initiated by an environment stressor or a viral infection that weakens pulmonary defense mechanisms, often allowing bacterial colonization (Yates, 1982; Griffin et al., 2010). Microorganism such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma* spp. are consistently regarded as the primary bacterial causes of BRD (Autio et al., 2007; Angen et al., 2009).

Historically, heifer calves have been raised from birth to weaning in individual hutches, to minimize direct contact and disease transmission, and fed a liquid diet of whole milk, non-saleable milk, or milk replacer twice daily at 10% of body weight (Drackley, 2008). This traditional system is the most common heifer-raising practice in the United States; approximately 75% of heifer-raising operations, despite the growing concern on animal welfare and labor costs associated with this practice (Kung Jr. et al., 1997; Chua et al., 2002; NAHMS, 2011). Additionally, the restricted amount of milk fed throughout the pre-weaning period can lead to reduced heifer growth and impact future productivity when compare to unrestricted or intensified feeding systems (Appleby et al., 2001; Soberon et al., 2012).

Group-housed heifer-raising operations typically group 10 to 25 calves in a single pen and provide unrestricted access to a liquid diet (whole milk, non-saleable milk, or milk replacer). To support unrestricted feeding, a common practice is to

conserve non-saleable milk or milk replacer by acidification (Nocek and Braund, 1986, Richard et al., 1988). This alternative system provides advantages to both the dairy producer and the calves, allowing a more natural behavior, improving weight gain and reducing labor requirements (Kung Jr. et al., 1997; O'Driscoll et al., 2006). However, a higher odds of respiratory disease for calves reared in groups when compared to single-calf pen was reported by Svensson et al. (2003), and rearing calves in groups increases the risk of pathogen transmission (Warnick et al., 1977).

Regardless of the heifer-raising system, antimicrobials are widely used to prevent and treat BRD. A common practice in feedlot herd health programs is the use of antimicrobial metaphylaxis to prevent BRD (Jim et al., 1999; Schunicht et al., 2002); the use of tilmicosin metaphylaxis on high-risk feedlot cattle was reported to reduce BRD incidence by 14% (Vogel et al., 1998). Additionally, studies have shown that administration of tilmicosin and florfenicol during the pre-shipping period in feedlot cattle can effectively inhibit *Mannheimia haemolytica* from colonizing the nasopharynx, suggesting that pre-shipment administration might reduce early onset of acute BRD (Frank and Duff, 2000).

As group-housed calf-rearing systems become more popular, it is expected that respiratory diseases will become more prevalent during the pre-weaning period. Therefore, the primary objective of this study was to evaluate the metaphylactic use of a synthetic long acting macrolide (tildipirosin) on the incidence of BRD, otitis, and survival in pre-weaned group-housed dairy calves that were fed unrestricted acidified milk. A secondary objective was to evaluate the effect of treatments on average daily gain (ADG).

MATERIALS AND METHODS

Animals and Facility

The study protocol was approved by the Institutional Animal Care and Use Committee of Cornell University (Protocol number 2013-0078). Assuming a type I error rate of 5%, a power of 80%, a two-sided statistical test, and a baseline probability of respiratory disease of 25%, a sample size of 250 calves per group was anticipated to detect a 10% reduction in the incidence of respiratory disease between the treatment and the control group.

The study was conducted from November of 2013 until June 2014 in a commercial dairy farm in upstate New York. Immediately after parturition, newborn calves were removed from the maternity pen and placed into a newborn pen bedded with dry sawdust and heated with heating lamps from November to March. All calves were fed approximately 4 L of raw pooled colostrum within 4 h of birth by esophageal feeder (Oral Calf Feeder Bag with Probe, Jorvet). Twice daily, newborn calves were moved from the newborn pen to a green-house type of barn with positive ventilation composed of 27 identical group-pens (70 m^2) bedded with straw. Twenty-five calves were placed in each pen; all calves remained in the same pen from day one of life until weaning (65 days of life). Birth weight and weight at weaning of all heifer calves were measured using a portable scale (Waypig-15, Vittetoe Inc.).

Blood samples were collected on day 3 of life for measurement of serum immunoglobulin-G using an ELISA kit (Bethyl Laboratories). Calves were fed unrestricted, acidified, non-saleable milk. Non-saleable milk was harvested twice daily and kept inside a stainless-steel tank at 5^{o}C until acidification process. The

acidification was performed inside the sealed stainless-steel tank where the non-saleable cold milk (5^{o}C) was constantly mixed with organic acid (formic acid 20% vol/vol) until a pH of 4.5 was reached. Acidified milk was kept for 72 hours inside the stainless-steel tank after the acidification process. After 72 hours, acidified milk was then directed to a smaller stainless-steel tank responsible for heating (18.5^{o}C) and supplying each pen-feeder with constant acidified milk. Each feeder was comprised of 6 nipples for a pen of 25 calves. All calves in this study were weaned by reducing the time of milk availability starting on day 55. After day 55 of life, the system was automatically set-up to reduce milk availability; gradual reduction of time was performed for 10 days until the complete absence of milk at 65 days of life.

Treatment administration

All heifer calves were randomly enrolled at birth into one of three treatment groups: 1) control group receiving no treatment (CTR), 2) one antibiotic injection administered at 10 days of life (M1), or 3) two antibiotic injections at 10 days and 35 days of life (M2). Randomization to each treatment group was performed a priori using random function in Excel (Microsoft). For this study, a synthetic long acting macrolide, tildipirosin (Zuprevo, Merck Animal Health), was the antibiotic of choice for the metaphylactic approaches due to its fast absorption and distribution to lung tissue (Menge et al., 2012).

The label for tildipirosin use includes the control of respiratory disease in beef and non-lactating dairy cattle at high risk of developing BRD. Under the Animal Medicinal Drug Use Clarification Act, its extra-label use as a metaphylactic

medication for control of otitis is justified as no other medications are labeled for this use in dairy calves.

Case definition

Bovine respiratory disease was defined when the following clinical signs were detected in a calf: cough, rectal temperature >39.5 °C, increased cranioventral lung sounds, or wheezes. Otitis was defined by observation of ear pain evidenced by head shaking, scratching or rubbing the ears, epiphora, ear droop, or signs of facial nerve paralysis, with or without fever (rectal temperature >39.5°C). The calf barn was visited daily by the research team; each pen was visually inspected to evaluate calf behavior (animal posture, feeding, and interaction with herd mates). If any abnormal behavior was detected, the animal was restrained inside the pen and a complete physical exam was performed. Following the diagnosis of BRD or otitis, animals were treated according to farm protocols.

Statistical Analysis

Descriptive statistics were performed using the FREQ, MEANS, and UNIVARIATE procedures of SAS (SAS Institute INC., Cary, NC). Differences in initial body weight, serum IgG concentration at 3 days of life, diarrhea events before10 days of age, proportion of calves born to primiparous cows, calving location (free-stall or maternity), and parturition difficulty (assisted or non-assisted) were evaluated between treatment groups.

Four different Cox Proportional Hazard models using the proportional hazard

regression procedure in SAS were used to evaluate the effect of metaphylaxis on calf survival, BRD, otitis, and the combined incidence of BRD and otitis. The independent variables offered were: treatment group (CTR, M1, and M2), birth weight quartile, serum IgG, diarrhea events before 10 days of life, dam parity (first, second, and third or greater lactation), calving location (free stall or maternity), and parturition difficulty (assisted or non-assisted). Treatment was the only variable forced into the models.

For all four Proportional Hazard Models, the final model was built using a backward stepwise selection of independent variables with a *P*-value < 0.05. Two-way interactions between remaining effects (including treatment) were tested and remained in the model if the *P*-value of < 0.05 was observed. To account for the effect of cluster, proportional hazard models were fitted using pen as a cluster (ID statement in SAS). Models proportional hazard assumptions were assessed based on a Kolmogorov-type Supremum test (ASSESS statement in SAS).

The effect of treatment and disease on ADG was evaluated by two similar general linear models using the MIXED procedure of SAS. Least square means and respective standard error of the mean were estimated for all categorical main effects. Pen was used as a random effect in both models. Average daily gain was calculated by subtracting the body weight at weaning from the birth weight and subsequently dividing by the total days from birth to weaning.

To evaluate the effect of treatment on ADG, the model was fitted to the data using the following independent variables: treatment group (CTR, M1, and M2), serum IgG, diarrhea events before 10 days of life, dam parity (first, second, and third or greater lactation), calving location (free stall or maternity), and parturition difficulty

(assisted or non-assisted). To evaluate the effect of disease (BRD and otitis) on ADG, the model was fitted to the data using the following independent variables: BRD (yes or no), otitis (yes or no), serum IgG, diarrhea events before 10 days of life, dam parity (first, second, and third or greater lactation), calving location (free stall or maternity), and parturition difficulty (assisted or non-assisted).

For both models, biologically meaningful interactions were offered and retained in the model when a *P*-value < 0.05 was observed. Residual distribution was assessed for homoscedasticity and normality. Statistical significance was declared at $P \leq 0.05$ and statistical tendencies declared at $0.05 < P \leq 0.10$.

RESULTS

A total of 795 Holstein heifer calves were enrolled into the study. Descriptive statistics regarding calf- and dam-related events are presented in **Table 5.1**.

Results of Cox proportional hazard models regarding effect of treatment group on mortality, BRD, otitis, and combined incidence of BRD and otitis are in **Table 5.2**. Mortality was not significantly different between treatment groups ($P = 0.21$). A tendency to have lower hazard of BRD was observed for M1 (HR = 0.68, $P = 0.07$) when compared to CTR group. Calves in M2 group also tended to have a lower hazard of BRD when compared to CTR group (HR = 0.70, $P = 0.09$). Additionally, a significantly lower hazard of having the combine BRD and otitis events was observed for M1 (HR = 0.70, $P = 0.009$) and M2 (HR = 0.72, $P = 0.01$).

Table 5.1: Descriptive statistics regarding number of calves, calves born from primiparous dams, assisted parturition, calving location, failure of transfer of passive immunity (serum IgG < 1,000 mg/dL), incidence of diarrhea before 10 days of life, serum IgG, and calf birth weight in each treatment group for 795 calves.

	CTR	M1	M2	P-value
n	*257*	*265*	*273*	
		Percentage		
Calves born to primiparous	48.6	49.8	49.4	0.87
Parturition assistance	12.4	9.8	9.9	0.45
Maternity pen calving	88.5	89.2	89.1	0.55
FTP	5.6	6.0	7.7	0.75
Incidence of diarrhea	12.3	12.9	11.9	0.83
		Mean (SEM)		
Serum IgG, mg/dL	1,56 (33.8)	1,49 (32.8)	1,56 (31.7)	0.27
Birth weight, kg	38.2 (0.26)	38.5 (0.26)	38.0 (0.25)	0.36

CTR, control group
M1, tildipirosin metaphylaxis at 10 days of life
M2, tildipirosin metaphylaxis at 10 and 35 days of life
FTP, Failure of transfer of passive immunity

Table 5.2: The effect of tildipirosin metaphylaxis on survival, bovine respiratory disease (BRD), otitis, and combined incidence of BRD and otitis in 795 pre-weaning dairy heifers were evaluated by 4 similar Cox`s proportional hazard models.

		Hazard Ratio (95% Confidence Limits)	P-value
	CTR	*Ref.*	
Mortality	M1	0.51 (0.30 – 1.13)	0.11
	M2	0.62 (0.28 – 1.29)	0.21
	CTR	*Ref.*	
BRD	M1	0.68 (0.47 – 0.97)	0.07
	M2	0.70 (0.49 – 1.01)	0.09
	CTR	*Ref.*	
Otitis	M1	0.85 (0.67 – 1.23)	0.34
	M2	0.80 (0.62 – 1.15)	0.30
	CTR	*Ref.*	
Combined BRD and Otitis	M1	0.70 (0.58 – 0.95)	0.009
	M2	0.72 (0.60 – 0.98)	0.01

CTR, control group
M1, tildipirosin metaphylaxis at 10 days of life
M2, tildipirosin metaphylaxis at 10 and 35 days of life

Metaphylaxis did not affect ADG (P = 0.55, **Table 5.3**). However, lower ADG were observed for calves born from primiparous dams (0.62 kg/day, ± 0.01, P < 0.001) and calves born weighing less than 35.8 kg (0.60 kg/day, ± 0.02, P = 0.01).

Table 5.3: The effect of tildipirosin metaphylaxis on calf average daily weight gain (ADG, kg/day) in 795 group-housed Holstein dairy heifers raised under unrestricted acidified non-saleable milk. Data are presented as least square means (LSM) and standard error of the mean (SEM).

		ADG (kg/day)		
		LSM	SEM	*P*-value
Metaphylaxis	CTR	0.63	0.01	
	M1	0.64	0.01	0.55
	M2	0.65	0.01	
Parity of the dam	1st	0.62	0.01	
	2nd	0.62	0.02	< 0.001
	3rd	0.69	0.02	
Birth weight, kg	<35.8	0.60	0.02	
	35.8 to 38	0.65	0.02	0.01
	38 to 40.8	0.65	0.02	
	>40.8	0.66	0.02	

CTR, control group
M1, tildipirosin metaphylaxis at 10 days of life
M2, tildipirosin metaphylaxis at 10 and 35 days of life

The effect of disease on ADG was also evaluated (**Table 5.4**). Calves affected with BRD had a lower ADG when compared to calves not affected with BRD (P = 0.007). Calves affected with otitis also had impaired growth when compared to healthy calves ($P < 0.001$). Moreover, calves that were affected with both BRD and otitis had lower ADG during the pre-weaning period ($P < 0.001$).

Table 5.4: General linear mixed models evaluating the effect of disease (bovine respiratory disease and otitis) on calf average daily weight gain (ADG, kg/day) in 795 group-housed Holstein dairy heifers raised under unrestricted acidified non-saleable milk. Data are presented as least square means (LSM) and standard error of the mean (SEM).

		ADG (kg/day)		*P*-value
		LSM	SEM	
Birth weight, kg	<35.8	0.58	0.02	0.004
	35.8 to 38	0.64	0.02	
	38 to 40.8	0.63	0.01	
	>40.8	0.66	0.02	
Parity of the dam	1st	0.59	0.01	0.001
	2nd	0.62	0.02	
	3rd	0.67	0.02	
Bovine respiratory disease	Affected	0.60	0.02	0.007
	Not affected	0.65	0.01	
Otitis	Affected	0.60	0.02	0.001
	Not affected	0.66	0.01	

DISCUSSION

Two metaphylactic approaches were evaluated in a commercial dairy farm where dairy heifer calves were raised in group-pens of 25 calves per pen and fed unrestricted, acidified, non-saleable milk. Metaphylaxis treatments were performed using a semi-synthetic macrolide (tildipirosin) injected at 10 days of life (M1) and a second group receiving two injections one at 10 and another at 35 days of life (M2). Metaphylaxis treatments had significantly lower hazard of the combined events of BRD and otitis during the pre-weaning period. However, only a tendency was observed when only BRD was evaluated, and no effect of treatment was observed for calves affected only with otitis. Additionally, metaphylaxis treatments did not affect ADG during the pre-weaning period.

The majority of the research regarding metaphylactic approaches against BRD has been conducted in feedlot cattle where the incidence of BRD fluctuates between 20% and 50% (Booker et al., 2007; Menge et al., 2012). However, lower incidences were observed in studies conducted on dairy heifers which ranged from 10% to 25% during the first 3 months of life (Sivula et al., 1999; Virtala et al., 1999; Bach et al. 2011).

In contrast to the extensive peer-reviewed literature regarding metaphylaxis approaches in feedlot cattle, to the best of our knowledge, only one study evaluating antimicrobial metaphylaxis has been conducted on pre-weaned dairy calves. Stanton et al. (2013) conducted a study on a heifer-raising facility to evaluate the effect of metaphylaxis (tulathromycin) at 3 (±2) days of life on the incidence of BRD. In that study, Stanton et al. (2013) reported that metaphylactic treatment had no effect on

BRD (only 2% of calves in the treatment group were affected with BRD and 3% in the control group). It is important to highlight that in the study conducted by Stanton et al. (2013), heifer calves originated from 5 different commercial dairy farms and once in the heifer raising facility, calves were individually allocated in 1 out of 6 naturally ventilated barns or in 47 individual outdoor hutches and fed 4L/feeding of milk replacer twice daily.

Similarly, tildipirosin metaphylaxis in the present study had no effect on BRD incidence during the pre-weaning period in Holstein heifer calves reared under an unrestricted, acidified, non-saleable milk regime and in groups of 25 calves per pen. Likewise, metaphylaxis had no effect on otitis prevention during the pre-weaning period in the present study. Otitis was diagnosed in 33.5%, 32.4%, and 30% of the calves enrolled in the CTR, M1, and M2 groups, respectively. Stanton et al., (2013) reported a significant effect of tulathromycin metaphylaxis on unilateral and bilateral ears droop reduction when compared to control calves.

Otitis and BRD are frequently concomitant diseases, sharing common risk factors and similar pathogens (Yeruham et al., 1999; Francoz et al., 2004) such as *Mycoplasma bovis* (Lerner et al., 2014), *Haemophilus somnus* (Walz et al., 1997), *Pasteurella multocida* (Nation et al., 1983), and *Mannheimia haemolytica* (Jensen et al., 1983). In the current study, although no effect on BRD or otitis was found with metaphylactic treatment, a protective effect was observed for the combined incidence of BRD and/or otitis during the pre-weaning period, likely due to early protection against some of the common pathogens associated with otitis and BRD.

There are many risk factors reported to be associated with BRD such as group

size (Svensson et al. 2006), birth season (Dennis, 1986), colostrum management (Donovan et al. 1998), and dam parity (Perez et al., 1990). Lombart et al. (2007) reported that calves born from severe dystocia had higher odds of receiving a treatment for BRD during the first 120 days of life. Additionally, a recent study evaluated the association between several risk factors with an increased risk of BRD in 19 herds in Canada and USA. The risk factors that significantly increased BRD incidence were herd-level incidence of BRD, season of birth (31.8% during the winter and 15.2% during the summer), navel dipping, other diseases before 2 weeks of age, failure of transfer of passive immunity, and manual control of temperature in pre-weaning housing (Windeyer et al., 2014). In the present study, for all the models evaluating the effect of treatment on BRD and otitis morbidity as well as mortality, neither calving assistance, parity of the dam, nor calving location were significantly associated with increased risk of BRD or mortality.

A prospective observational cohort study conducted by Sivula et al. (1999) reported an association between calves with failure of transfer of passive immunity (serum IgG concentration < 800 mg/dL) and an increased risk of mortality and BRD. In the present study, failure of transfer of passive immunity was defined as a serum IgG concentration at 3 days of life < 1,000 mg/dL and was below 8% in all treatment groups. Besides the method of assessing adequate transfer of passive immunity on newborn calves, using total serum solids (> 5.5 g/dL) or enzyme-linked immunosorbent assay (IgG > 1000mg/dL), numerous studies have reported adverse effects of failure of transfer of passive immunity on heifer mortality, health, and post-weaning performance (Faber et al., 2005; Priestley et al., 2013; Windeyer et al., 2014).

However, in this study, failure of passive transfer was not found to be significantly associated with mortality, morbidity, and ADG during the pre-weaning period.

In the present study, metaphylaxis did not affect ADG during the pre-weaning period but dam parity and birth weight quartiles did. Calves born to primiparous cows and calves in the lowest birth weight quartile had the lowest ADG, while calves born to cows at third or greater parity and calves in the highest birth weight quartile had the highest ADG during the pre-weaning period. Sivula et al. (1996), reported comparable results as ADG significantly increased with parity of the dam. Similarly, a study on 2,491 calves followed to 180 days of life conducted by Simensen and Norheim (1983) reported calves born to first parity dams had the smallest chest girth at 180 days of life while calves born to third parity or greater dams had the largest measurements. Parity can also affect the birth weight, as a study reported birth weights of female calves born to second and third lactation cows to be approximately 8% heavier when compared to primiparous cows (Kertz et al., 1997). Birth weight of heifer calves was elsewhere reported to impair ADG, where calves lighter at birth gained significantly less weight by weaning (Teixeira et al., 2013).

Here we observed that calves affected with BRD had significantly lower ADG during the pre-weaning period compared to healthy calves. The effect of calfhood disease (diarrhea, septicemia, and respiratory disease) has been consistently associated with lower ADG in many studies. Virtala et al. (1996) reported that calves diagnosed and treated for BRD had lower ADG in the first month of life compared to healthy calves, and the negative effect of calf morbidity (BRD and diarrhea) on growth rate from birth to 90 days of life was reported by Lundborg et al. (2003). Ganaba et al.

(1995) reported that calves diagnosed with diarrhea or respiratory disease during their first 2 weeks of life had lower growth rates, and Donovan et al. (1998) reported that diarrhea, septicemia, or BRD before weaning reduced the weight of 6 month-old calves by 4.8 to 10.6 kg.

Similar to BRD, we found that significantly lower ADG were observed for calves affected with otitis when compared to healthy calves during the pre-weaning period; calves affected with otitis had on average a 60.1 g/day reduction in weight gain during the pre-weaning period compared to healthy calves. This effect has varied in previous studies. Stanton et al. (2013), when evaluating the effect of disease in ADG during the pre-weaning period, reported a 50 (±20) g/day difference between calves affected with unilateral ear droop compared to calves without ear droop; however, the ADG was not different between calves affected with bilateral ear droop when compared to calves without ear droop. Contrarily, Pardon et al. (2012), when evaluating the impact of BRD, diarrhea, otitis and arthritis on carcass traits in white veal calves, found no significant association between otitis and hot carcass weight.

CONCLUSIONS

Tildipirosin metaphylaxis treatment had no effect on mortality, otitis, and ADG during the pre-weaning period; however, a tendency to have lower hazard of BRD was reported for calves receiving metaphylaxis treatment. When evaluating the combined incidence of BRD and otitis, tildipirosin metaphylaxis significantly reduced disease incidence. Long-acting antibiotic metaphylaxis could be strategically used to decrease the combined incidence of BRD and otitis in high-risk group-housed replacement heifers during the pre-weaning period.

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CHAPTER 6

THORACIC ULTRASOUND ASSESSMENT OF LUNG CONSOLIDATION AT WEANING IN HOLSTEIN DAIRY HEIFERS: REPRODUCTIVE PERFORMANCE AND SURVIVAL

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ABSTRACT

The objective of this study was to determine the association of lung consolidation at weaning with later reproductive performance and survival. Ultrasonography of the lungs was performed at 60 d of life in recently weaned Holstein heifer calves from a single farm in New York State. Thoracic screening covered the right 2nd through 10th and left 3rd through 9th intercostal spaces and was performed using a 6.2 MHz linear transducer. Each calf was classified as not having lung consolidation (NC; hyperechoic line with reverberation artifact with or without comet tail) or with lung consolidation (LC; any detectable heterogeneous hypoechoic area). A total of 613 heifer calves were enrolled in the study, with 489 (79.8%) classified as NC and 124 (20.2%) classified as LC.

No difference in mortality was observed from 60 to 350 d of life between heifers with lung consolidation (1.6%) and without lung consolidation (2.0%). Six hundred and one nulliparous Holsteins became eligible for insemination at 350 d of life; there was a higher hazard of being removed from the herd between 350 d of life and first calving for heifers with lung consolidation at weaning (hazard ratio = 4.7, 95% confidence interval = 2.1 to 10.7). Additionally, heifers without lung consolidation tended to have improved pregnancy to first AI (62.0%) compared to heifers with lung consolidation (52.5%). Overall reproductive performance was also affected as heifers with lung consolidation at 60 d of life had a lower hazard of pregnancy compared to those without lung consolidation (hazard ratio = 0.7, 95% confidence interval = 0.6 to 0.8).

From 601 animals that the breeding period, 565 entered the milking herd and

were followed during the first 3 months of lactation. No differences in weekly average milk production were observed between animals with or without lung consolidation at weaning.

Our results show that heifers with lung consolidation at weaning were less likely to get pregnant and more likely to be culled before their first parturition than heifers without lung consolidation; this difference did not continue into first lactation milk production, risk of culling, or reproductive performance.

INTRODUCTION

Bovine respiratory disease (BRD) affects approximately 16% of pre-weaned heifers in the United States, of which 90.2% of BRD affected pre-weaned heifers are treated with antibiotic (USDA, 2012), leading to impaired survival and reproductive performance (Stanton et al., 2012). There is currently no gold standard for BRD diagnosis, and researchers have developed two useful respiratory scoring charts to assist with detection and treatment of BRD (McGuirk, 2008; Love et al., 2014). In addition to these subjective measures, thoracic ultrasound has been proposed as a useful calf-side tool to improve quantitative diagnosis of BRD (Buczinski et al., 2013; Buczinski et al., 2014). Studies assessing the sensitivity and specificity of thoracic ultrasound in diagnosing BRD, where BRD was confirmed by necropsy, reported a sensitivity of 86% to 94% and a specificity of 98% to 100% (Rabeling et al., 1998; Ollivett et al., 2015). It is important to note that in the Rabeling study only clinical cases were evaluated and that the Ollivett study had a small number of animals. Thoracic ultrasound has been proposed as a calf-side tool to aid BRD diagnosis, detect pulmonary lesions, and reduce use of antimicrobials (Jung and Bostedt, 2004; Ollivett et al., 2011).

The extent to which lung lesions, confirmed at slaughter, can impair subsequent productivity has been assessed by multiple studies in the feedlot cattle. These studies found lower weight gain during feeding, a high incidence and lower weight gain in subclinical (never diagnosed or treated) BRD animals, and economical losses ranging from $1.79 per animal entering the feedlot, to approximately $54 for animals presenting lesions at slaughter and recurrent BRD treatments (Thompson et

al., 2006; Schneider et al., 2009). In contrast, results regarding the impact of pulmonary lesions on milk production and reproductive performance of dairy animals are scarce. To our knowledge, only one study has been conducted that correlates lung lesions in dairy cattle to performance (Adams and Buczinski, 2016). In this study, lung consolidation was assessed using thoracic ultrasonography in three-month old Jersey heifers. They reported that heifers with extensive consolidation had a higher risk of being removed from the herd between 80 and 330 d of life but did not find a significant impact of lung score to age at first calving. While few studies have evaluated pulmonary lesions, more have reported detrimental effects of early life diagnosis of BRD on performance through the first lactation, higher risk of herd removal, and delayed age at first calving (Bach, 2011; Stanton et al., 2012).

Given the limited knowledge surrounding pulmonary lesions in dairy heifers and their association with production outcomes, our objective was to explore the consequences of lung consolidation in dairy heifers at weaning with subsequent survival and reproductive performance. For this prospective cohort study, we hypothesized that heifer calves with lung consolidation at weaning would have higher age at pregnancy and a higher culling risk when compared to herd mates without lung consolidation. A secondary objective was to investigate the association of lung consolidation at weaning with weekly milk average, risk of culling, and risk of pregnancy to first service within the first 3 months of first lactation.

MATERIALS AND METHODS

The study was conducted on a commercial dairy farm located near Ithaca, New York from November 2013 until February 2014. The study was approved by the Institutional Animal care and Use Committee of Cornell University (protocol number 2013-0076).

Pre-weaning Management

This study was conducted in a commercial dairy farm milking approximately 3,700 Holstein cows, near Ithaca, NY. Newborn Holstein dairy heifers were fed 4 L of pooled pasteurized colostrum via esophageal tube within 4 h after birth and moved daily from the maternity pen to the pre-weaning calf barn. Pre-weaning heifers were housed in a green-house type barn with positive ventilation composed of 10 identical group-pens (85 m^2) bedded with straw. Twenty-five calves were placed in each pen; all calves remained in the same pen from d one of life until weaning at 60 d of life.

All heifer calves were fed unrestricted, acidified, non-saleable milk. Acidification was performed inside a sealed stainless-steel tank where the non-saleable cold milk (5^{o}C) was constantly mixed with organic acid until a pH of 4.5 was reached. Acidified milk was kept for 72 h inside the stainless-steel tank after the acidification process was finished. Milk was then directed to a smaller stainless-steel tank responsible for heating (18.5^{o}C) and supplying each pen-feeder with constant acidified milk. Each feeder was comprised of 6 nipples for a pen of 25 calves. All calves in this study were weaned by reducing the time of milk availability starting on d 55; a gradual reduction of time was performed for 5 d until the complete absence of

milk at 60 d of life.

Lung Ultrasonography

One member of the research team was responsible for performing lung ultrasonography on all calves at 60 d of life. A structure was built using stainless steel bars in a gate-like "U" layout (3 parts); the structure was fixed inside each pen (1.5m x 0.75m) by latching one end to the gate pen which allowed for single calf isolation where ultrasonographic exams were performed. This structure was washed and used for the next pen by the time of thoracic ultrasound examination. Thoracic ultrasound examinations were carried out using an Ibex-pro device with a 6.2 MHz linear transducer (E.I. Medical Imaging). Examination of the lung areas was performed by screening dorsal to ventral intercostal spaces from the right 2^{nd} through 10^{th} and left 3^{rd} through 9^{th} intercostal spaces. The first intercostal space of the right side was not included in the examination because examiner could not consistently reach 1^{st} intercostal space for all the calves examined. Calves were not shaved in any area on the thorax; to achieve better contact and imaging quality, 70% isopropyl alcohol was applied to the haired areas under examination.

The scoring procedure used in this study was an adaptation from Ollivett et al., 2011. Thoracic ultrasound was performed on each hemithorax for all calves and a 2 point-scale assigned: no lung consolidation (NC) or with any detectable lung consolidation (LC). Animals received one score based on the combined exam of the right and left thoracic area. Each exam required approximately 5 min per calf. Heifers were classified as NC if no abnormalities were detected on thoracic ultrasound, i.e.

well-ventilated peripheral lung tissue (hyperechoic line with reverberation artifact) or comet-tails artifacts observed in one or multiple lobes (hyperechoic vertical lines originated from the aerated lung surface). Heifers were classified as LC if a detectable consolidation was observed, meaning any size consolidation (heterogeneous hypoechoic area without the clear line of the pleural surface) detected in one or more lung lobes (**Figure 6.1**).

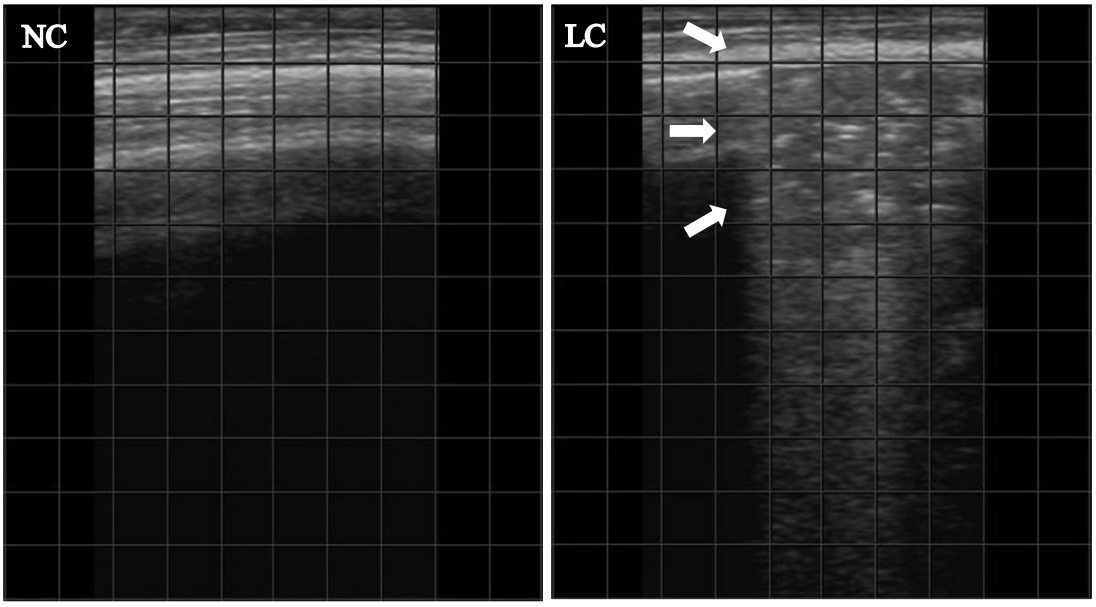

Figure 6.1: Ultrasonographic evidence of heifers with no consolidation (NC; well-ventilated peripheral lung tissue represented by hyperechoic line with reverberation artifact or with comet-tails artifacts observed in one or multiple lobes represented by hyperechoic vertical lines originated from the aerated lung surface) and heifers with detectable consolidation (LC; any detectable consolidation represented by a heterogeneous hypoechoic area without the clear line of the pleural surface was detected in one or more lung lobes). Each square is 1 cm^2.

Post-weaning Management

After thoracic ultrasound, heifers were moved to a post-weaning pen. Weaned heifer pens were fed TMR daily to maintain a weight gain of approximately 0.9 kg/d (NRC, 2001). Vaccination against infectious bovine rhinotracheitis virus, bovine viral diarrhea virus (types 1 and 2), bovine respiratory syncytial virus, parainfluenza-3 virus, and clostridial diseases were performed at 3 months of age (Vista 5 SQ and Covexin 8, Merck Animal Health). Heifers were moved weekly to breeding pens around 350 d of life. Once in the breeding pens, heifers were allowed 30 d of artificial insemination upon estrus detection before receiving an injection of prostaglandin followed by estrus detection. Heifers not detected in estrus were given a repeat prostaglandin injection every 2 weeks until 550 days of life. Pregnancy diagnoses were performed by veterinarians for inseminated animals between 35 and 41 d post-AI. Pregnancy diagnoses were confirmed by a re-check performed between 61 and 74 d post-AI. Starting at approximately 240 d of gestation, heifers were moved weekly from the breeding pens to a close-up pen where animals were more closely monitored by farm employees. Pregnant animals detected in stage 1 or 2 of parturition were moved to the maternity pen. After calving, primiparous cows were milked at the maternity pen and then moved to a fresh cow pen within 6 hours of first milking. Primiparous fresh cows were milked 3 times per d in a 100-stall rotary parlor. All lactating animals were offered TMR formulated to meet or exceed the National Research Council nutrient requirements (NRC, 2001) for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% fat corrected milk.

A voluntary waiting period of 50 d was used for primiparous cows. The

reproductive management for first lactation cows utilized a combination of Presynch, Ovsynch, Resynch, and detection of estrus, with approximately 30% of cows bred via timed artificial insemination (**TAI**) and the remainder bred after detection of estrus solely by activity monitors (Alpro, DeLaval). Briefly, the protocol for first insemination utilized two injections of prostaglandin administered 14 d apart for pre-synchronization, followed by the Ovsynch-56 protocol 7 d later to facilitate TAI. Animals detected in estrus after the second prostaglandin injection through the start of the Ovsynch protocol were inseminated. Pregnancy diagnoses for primiparous cows were performed for inseminated animals between 35 and 41 d post-AI and confirmed by a re-check performed between 61 and 67 d post-AI.

Animals were followed until the first 90 DIM. Post-partum diseases were diagnosed by trained farm employees; retained placenta was noted for animals that retained fetal membranes >24 hours after parturition, metritis was recorded for animals with fetid watery red-brown uterine discharge with or without fever within 21 d post-partum, and clinical mastitis noted as animals with at least one quarter presenting abnormal milk. Treatments were performed according to farm protocols.

Statistical Analyses

A priori sample size calculation was performed based on a 10% difference in survival rate between heifers without lung consolidation (0.95) and heifers with lung consolidation (0.85). Expecting that lung consolidation would be detected by the end of the weaning period in one out of 10 heifers, with a probability of Type I error of 0.05 and a power of 0.80, a total of 600 heifers were enrolled in this study.

The data retrieved from the farm's computer software (DairyComp 305 Valley Agricultural Software) included: age at first breeding, age at conception (defined after pregnancy diagnosis confirmation at approximately 60 d of gestation, back calculated to the number of d from birth to the d of conception), removal from herd (including death and animals that were sold), pregnancy risk to first service (percent of animals pregnant to the first artificial insemination), pregnancy length, age at first parturition, abortions (gestation length <260 d), twins, stillbirth, assisted or non-assisted parturition, and offspring birth weight (measured before colostrum feeding by farm employees using a dedicated scale; Waypig Digital 500, Raytec). First lactation data regarding average weekly milk production were collected until 90 DIM. Data regarding post-partum diseases (retained placenta, metritis, and clinical mastitis) were also retrieved from farm records.

Descriptive statistics regarding differences between lung score category (NC versus LC) and the normally distributed continuous variables (age at first breeding, age at first calving, gestation length, and offspring weight at birth) were performed using two-sided t-tests. Chi-square tests were used to compare the risk of pregnancy to first service, abortion, stillbirth, twinning, and assisted parturition between lung consolidation groups (TTEST and FREQ procedures in SAS; version 9.4, SAS Institute Inc.).

To assess the association of lung score with survival and reproductive performance, two similar Kaplan-Meier time-to-event models were fitted using the LIFETEST procedure in SAS. For the culling analysis animals were censored on the d of herd removal regardless of reason (animals that died or were sold) or right-censored

on the d of parturition. For the reproductive performance analysis (n = 601), heifers were censored at age at conception or right-censored at time of herd removal for animals that were never confirmed pregnant. The last pregnant animal calved at 840 d of life, which was then considered the end of the evaluation period. Since no animals were removed from the herd after 550 d, the time-to-event was arbitrary limited to 740 d of life. Kaplan-Meier plots were created using MedCalc (Version 16.4.3, MedCalc Software, BVBA).

For animals entering the milking herd (n = 565), differences in the number of animals in each cohort based on lung consolidation, proportion of primiparous animals diagnosed pregnant at first service (n = 546), incidence of retained placenta (n = 565), metritis (n = 565), and clinical mastitis (n = 565) tests were performed using the FREQ (chi-square test) and TTEST (two-sided t-test) procedures in SAS. Additionally, two similar Kaplan-Meier models were used to evaluate the association of lung ultrasonography performed at 60 d of life with primiparous reproductive performance (n = 546) and survival for the first 90 DIM (n = 565).

Weekly milk production data were collected for the first 90 DIM from all the cows entering the milking herd (n = 565). Average weekly milk production for the first 90 DIM was assessed using repeated-measures ANOVA with the MIXED procedure of SAS and an autoregressive covariance structure. Animal within lung consolidation group was treated as a random effect, and thoracic ultrasound score (NC or LC) and week in milk (1 to 12) were treated as covariates. Least square means and standard errors (SE) were estimated and reported. Model fit was assessed by visually evaluating the distribution plot of the Studentized residuals. For this model,

Bonferroni correction for multiple comparisons was used. Statistical significance was declared at $P \leq 0.05$, and statistical tendencies at $0.05 < P \leq 0.10$.

RESULTS

A total of 613 heifer calves were enrolled in the study, with 489 (79.8%) classified as NC and 124 (20.2%) classified as LC. From 60 to 350 d of life, 1.6% of animals in the LC group and 2.0% of animals in the NC group were removed from the herd ($P = 0.74$). Thus, the total number of animals remaining in the study at 350 d of life was 601.

For 601 animals entering the reproduction phase, survival was evaluated from 350 d of life to age at calving (**Figure 6.2**). The proportion of animals culled in the LC cohort was 15.6% and 3.5% for the NC cohort during this period. A higher hazard of death was observed in LC than NC heifers (hazard ratio (HR) = 4.7, 95% CI = 2.1 to 10.7, $P < 0.001$).

Additionally, LC heifers had a lower hazard of pregnancy when compared to NC heifers (HR = 0.7, 95% CI = 0.6 to 0.8, $P = 0.006$, **Figure 6.3**). No differences in age at first breeding were observed between the two cohorts. However, age at first calving was significantly lower in NC heifers than LC heifers ($P = 0.04$, **Table 6.1**). A tendency for lower pregnancy risk to first service was observed in LC heifers diagnosed with lung consolidation (52.5%) when compared to NC heifers (62.0%, $P = 0.06$).

From 601 heifers that entered the reproduction phase at 350 d of life, 565 pregnant heifers calved and entered the milking herd. No differences were observed

for any of the other variables: gestation length, stillborn, twins, abortions, assisted parturition, and offspring birth weight (**Table 6.1**).

Table 6.1: Thoracic ultrasonography was performed at 60 days of life and heifers were classified as; no lung consolidation (only clear pleural surfaces or comet tails were observed, solid line) or lung consolidation (any detectable consolidation in one or more lung lobes, dashed line). Table regarding age at first breeding, age at first calving, gestation length, offspring weight, percentage of pregnancy to first service, abortion, stillbirth, twins, and assisted parturition between heifer with and without lung consolidation.

	No lung consolidation Mean (SE)	Lung consolidation Mean (SE)	*P*-value
Age at first breeding, d	386.7 (0.6)	386.0 (1.0)	0.42
Age at first calving, d	679.8 (1.4)	687.4 (2.0)	0.04
Gestation length, d	277.6 (0.2)	277.2 (0.5)	0.48
Offspring weight, kg	37.5 (0.2)	37.4 (0.5)	0.76
Pregnancy to first service, %	62.0	52.5	0.06
Abortion, %	2.2	2.9	0.63
Stillbirth, %	6.9	9.1	0.12
Twins, %	2.7	2.5	0.69
Assisted parturition, %	17.3	20.3	0.35

No differences in survival were observed for primiparous cows between LC and NC animals between calving and the first 90 DIM. The hazard of culling was 1.0 (95% CI = 0.5 to 2.1, $P = 0.93$). Moreover, only 546 primiparous cows that entered the milking herd were alive at 50 DIM (50 d of VWP). No differences in reproductive performance were observed between LC and NC animals; the hazard of pregnancy was 1.0 (95% CI = 0.7 to 1.4, $P = 0.81$).

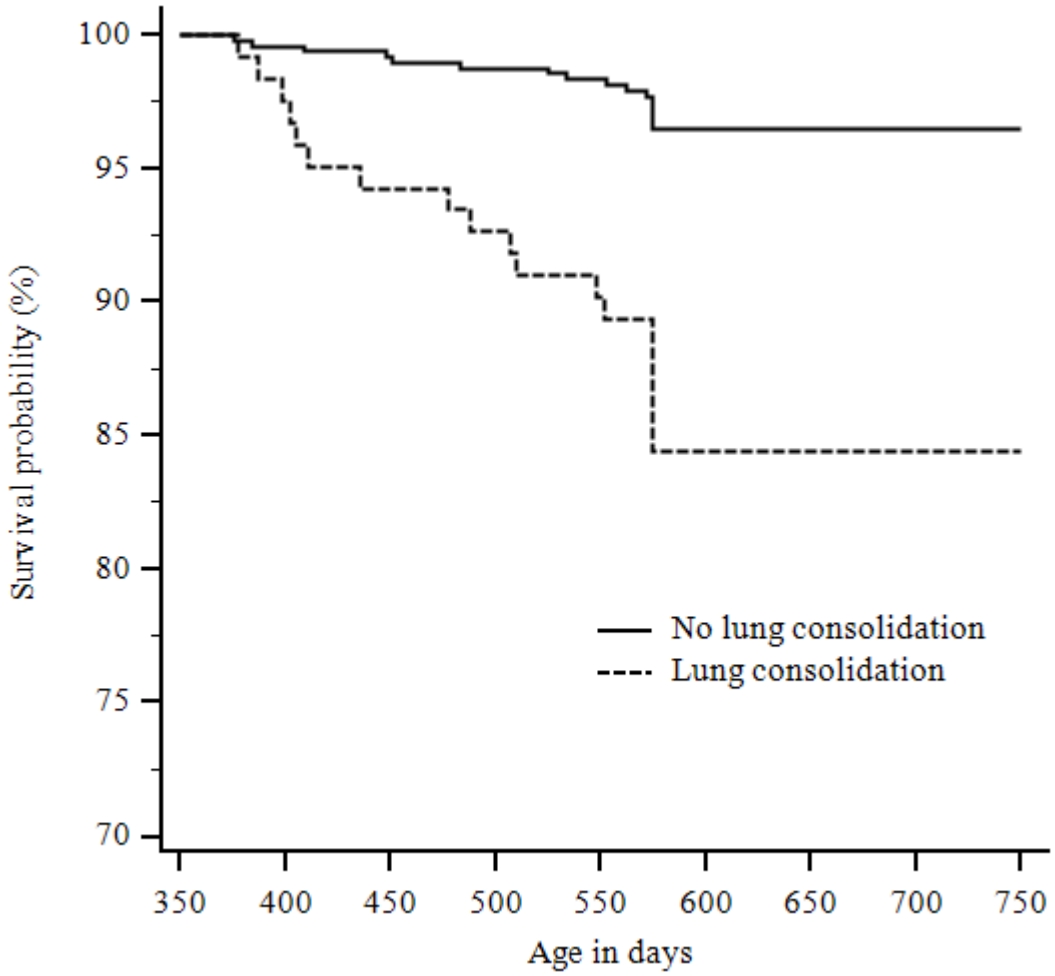

Figure 6.2: Kaplan-Meier analysis of time to culling in 601 nulliparous Holstein heifers that underwent lung ultrasonography evaluation at 60 d of life. Ultrasonographic lung score of each thoracic ultrasound was evaluated in a 2 point-scale defined as: no lung consolidation (only clear pleural surfaces or comet tails were observed, solid line) or lung consolidation (any detectable consolidation in one or more lung lobes, dashed line). Heifers with lung consolidation had a higher hazard of culling compared to heifers without lung consolidation (hazard ratio = 4.7, 95% CI = 2.1 to 10.7, $P < 0.001$).

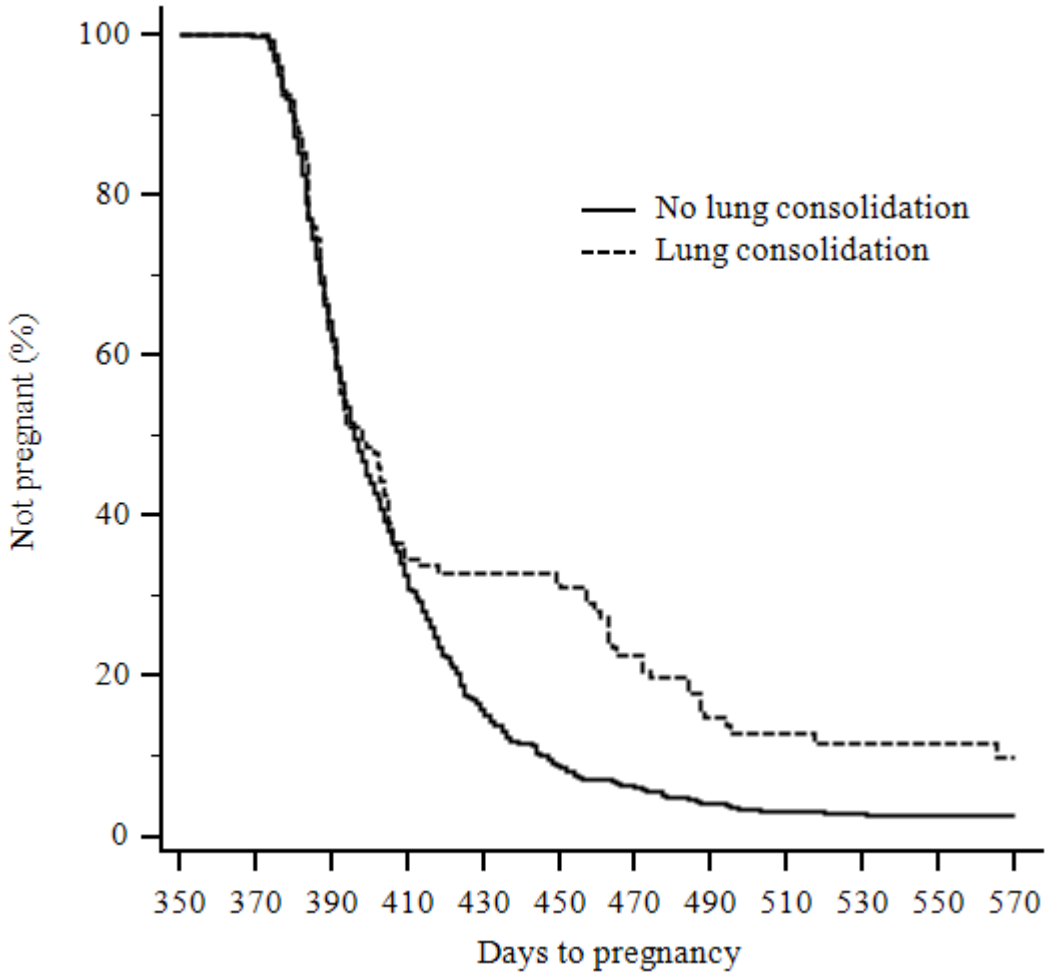

Figure 6.3: Kaplan-Meier analysis of time to pregnancy in 601 nulliparous Holstein heifers that underwent lung ultrasonography evaluation at 60 d of life. Ultrasonographic lung score of each thoracic ultrasound was evaluated in a 2 point-scale defined as: no lung consolidation (only clear pleural surfaces or comet tails were observed, solid line) or lung consolidation (any detectable consolidation in one or more lung lobes, dashed line). Animals were followed from 350 d of life until age of confirmed pregnancy or removal from the herd. Heifers with lung consolidation had a lower hazard of being pregnant compared to heifers without lung consolidation (hazard ratio = 0.7, 95% CI = 0.6 to 0.8, P = 0.006).

No differences between incidences of post-partum diseases or pregnancy to first service were observed for primiparous cows. For 565 pregnant heifers that calved, no differences were found regarding the incidence of retained placenta ($P = 0.15$) between LC (6.0%) and NC (2.7%), incidence of metritis ($P = 0.74$) between LC (32.2%) and NC (30.6%), and incidence of clinical mastitis ($P = 0.90$) between LC (6.1%) and NC (5.8%). For 546 primiparous cows, no difference between pregnancy to first service was observed ($P = 0.38$) between LC (30.2%) and NC (26.8%).

First lactation average weekly milk production data were collected. No differences were observed for the first 12 weeks of milk production between animals diagnosed with or without lung consolidation as heifers ($P = 0.73$).

DISCUSSION

To our knowledge, this is the first study evaluating the association of one-time thoracic ultrasound in replacement Holstein heifer calves and later performance. Although this is a single herd study, 613 heifer calves underwent thoracic ultrasound at 60 d of life in which 546 of these calves continued to be followed through 90 DIM. Heifers detected with lung consolidation at 60 d of life had a higher age at first calving, a higher hazard of death, and a lower hazard of pregnancy than heifers without lung consolidation. Additionally, a tendency for lower pregnancy to first service was reported for heifers with lung consolidation when compared to heifers without lung lesions. However, the effect of a one-time ultrasonographic evaluation at 60 d of life seemed to have detrimental effects only before animals entered their first lactation. No differences were observed for post-partum disease incidences (retained placenta, metritis, and mastitis), reproductive performance, or survival in the first 90 DIM between animals with lung consolidation or without consolidation at 60 d of life.

In a recently published study, 240 Jersey heifers had a single thoracic ultrasound at 96 ±6 d of life and were followed until age at calving or removal from the herd (Adams and Buczinski, 2016). Calves with at least one site of consolidation (≥ 6 cm), abscessation within the lung parenchyma, or significant pleural effusion (> 1 cm) had a higher chance of being removed from the herd. Interestingly, the survival of Jersey heifers with limited extent of lung consolidation (consolidation between 1 and 6 cm) was no different than heifers with no consolidation. In our study, heifers with no lung consolidation on thoracic ultrasound had better survival from 350 d of life to calving than calves showing any consolidation. The scoring system generated by

thoracic ultrasound in our study was simplified even further than others have proposed (Ollivett et al., 2011; Adams and Buczinski, 2016). Thoracic ultrasonography was performed in a stationary unit that allowed the examination of the right 2nd through 10th and left 3rd through 9th intercostal spaces. In the present study, researchers were unable to include the ultrasound data from the 1st intercostal space, this certainly decrease the researchers ability to detect possible consolidations present in the cranial aspect of the right cranial lobe, which can be inspected at the 1st and 2nd intercostal space on the right hemithorax (Ollivett et al., 2013).

Our observed outcomes for calves with lung consolidation at 60 d of life are in agreement with the deleterious effect of BRD detected before 90 d of age, specifically delayed age at first calving (Correa et al., 1988) and increased risk of death (Stanton et al., 2012). Additionally, poorer reproductive performance was reported for BRD treated calves before 60 d of life (Bach, 2011; Stanton et al., 2012). In the current study, because of the known subjectivity of BRD detection (Sivula et al., 1996), no attempt was made to retrieve farm data on BRD treatments. Two very useful charts are widely used as an on-farm tool to diagnose BRD (McGuirk, 2008; Love et al., 2014). Although chart driven systems are a good tool in helping caretakers to diagnose cases of BRD, they are still highly dependent of the ability of the caretaker to observe clinical signs; misclassification could lead to unnecessary use of antimicrobials and misleading data regarding BRD.

In the current study, Holstein heifers without lung consolidation at 60 d of life had 62.0% pregnancy to first service which is in agreement with data published elsewhere for Holstein heifers in United States (Ettema and Santos, 2004). However, a

tendency of lower pregnancy rate at first service (52.5%) was observed for heifers with lung consolidation. The reproductive performance results presented for nulliparous heifers might be attributed to a lower weight gain during the first 6 months as weight gain has previously been related to lung lesions in feedlot steers (Wittum et al., 1996). Delayed age at first calving and lower pregnancy risk at first service are frequently associated with suboptimal growth rates before 6 months of age (Brickell et al., 2009). However, no differences in age at first breeding between heifers with or without lung consolidation were observed in this study.

The thoracic ultrasonography technique used in our study can be performed in a relatively short time using a conventional linear probe and ultrasound. While no ante mortem calf-side gold standard tool to diagnose BRD exists, implementation of thoracic ultrasound should be considered by veterinarians in replacement heifer facilities; this practice can be used to detect heifers with higher risks of replacement failure. Different management practices can then be assigned to at-risk groups of heifers as needed, adjusting growth rates, pathogen screening, and selective use of antimicrobials.

CONCLUSIONS

As proposed by others that performed thoracic ultrasonography, the use of a calf-side tool to improve replacement heifer management should be further investigated using a larger number of animals and in different pre-weaning rearing systems. Although no detrimental effects of lung lesions at 60 d of life were observed for primiparous cows during the first 90 DIM into the first lactation, we found significant impacts for nulliparous heifers through a decrease in reproductive performance and an increase in culling risk.

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CHAPTER 7

FINAL CONSIDERATIONS

The aim of this dissertation was to enrich the body of studies regarding calfhood disease prevention and control as well as assess alternatives to standard practices to raise replacement dairy heifers. We evaluated alternatives to common methods used to reduce bacterial contamination of colostrum and non-saleable milk (Chapter One) and further evaluated if early life injectable trace mineral supplementation would improve dairy heifers' health and performance (Chapter Two). Neonatal enteritis or calf scour is commonly treated and prevented with the use of antimicrobials, and herein we tested if a natural product could be used to prevent water losses due to secretory diarrhea, decreasing the dehydration burden of scouring calves in a single pathogen challenge study (Chapter Three) and in a scenario mimicking farm conditions (Chapter Four). As group-housed calf-rearing systems have become more popular, respiratory disease has also become more prevalent during the pre-weaning period. Therefore, two metaphylactic approaches were tested in a randomized clinical trial to test if high-risk dairy heifers would benefit from such intervention (Chapter Five). Finally, we investigated if pulmonary lesions caused by early events of pneumonia could impair replacement heifers' productivity and reproductive performance (Chapter Six).

In chapter one, we conducted a block randomized clinical trial in a commercial dairy farm an attempt to test if ultraviolet light could be used as an alternative to heat treatment of colostrum and non-saleable milk. For this study, we hypothesized that the antimicrobial ultraviolet light properties could lead to decreased bacterial load (similar to heat treatment) without detrimental effects on immunoglobulins concentration (typical when using heat treatment). Data regarding incidence of diseases (respiratory

disease, otitis, and diarrhea) and body weight were recorded. Additionally, dam related events that could potentially affect calf performance (dystocia and health events around parturition) were also collected. In this first study, we observed that heat treatment was generally more effective than ultraviolet light in reducing bacterial count for both colostrum and non-saleable milk. More interestingly, we observed that ultraviolet light had a greater detrimental effect on the colostrum immunoglobulin content when compared to heat treatment of colostrum. From an animal perspective, calves fed colostrum treated with either ultraviolet light or heat had lower serum level of immunoglobulin isotype G when compared to calves fed raw colostrum. Animals receiving ultraviolet light and heat treated colostrum and non-saleable milk during the pre-weaning period presented similar growth, incidence of diseases, and mortality rates. Acknowledging that this study was conducted in a single and well-managed farm, we found significant results regarding calf performance that were not related to colostrum and milk treatments; dam's parity, metritis of the dam, and calf birth weight were significantly associated with calf survivability. Parity, calf birth weight, and parturition assistance were significantly associated with the incidence of diarrhea. Metritis and retained placenta of the dam were significantly associated with calf pneumonia. Future studies should be conducted to evaluate the immunological status of the pregnant cow and the immunological status its offspring. Association between dam`s health events in the post-partum and its offspring survival were found and were independent from colostrum, since pooled colostrum was used.

In chapter two, understanding the immunological challenges on a neonatal animal can be largely influenced by nutrient availability; we proposed to evaluate the

effect of supplementation of trace minerals on the development and function of the immune system. We hypothesized that supplementation of injectable trace mineral (containing 60 mg of zinc, 10 mg of manganese, 5 mg of selenium, and 15 mg of copper) would lead to an improvement in pre-weaning dairy heifers' immunological status, leading to decreased disease incidences and better growth performance during the pre-weaning period. For this study, we performed a randomized clinical trial using two commercial dairy farms and two different rearing systems (individual-housing fed restricted milk and group-housing fed unrestricted milk). Calves enrolled in this study were injected at three and thirty days of life. Immunological parameters, immune cell function, disease incidences, and body weight were measured at several points during the pre-weaning period. For both rearing systems, trace mineral supplementation was able to improve immune cell function (increasing leukocyte phagocytic ability), decrease incidence of diarrhea, and decrease incidence of combined respiratory disease and otitis. However, we could not observe a difference in calf body weight gain and mortality rates. More studies are needed to evaluate trace minerals levels in milk fed pre-weaned Holstein dairy calves to determine adequately when and which trace mineral should be supplemented.

In chapters three and four, we conducted two studies to test the efficacy of a natural product in reducing the dehydration caused by neonatal secretory diarrhea. This natural product, namely Crofelemer, has antisecretory properties that involve the inhibition of two distinct chloride channels on the luminal membrane of the intestine. In chapter three, we conducted a study to test if this natural product would aid calves under a single agent challenge. The microorganism of choice was enterotoxigenic

Escherichia coli known to mediate pathophysiological mechanisms of secretory diarrhea. During this study, fecal samples were collected to precisely quantify water content in feces. Data regarding fluid therapy intervention and body weight were also recorded. After challenged with enterotoxigenic *E.coli*, neonatal calves were treated twice daily consecutively for three days with a bolus of enteric-coated Crofelemer extract, or a non-enteric-coated bolus, or a placebo bolus. Calves suffering from secretory diarrhea in this study had significantly less water content in fecal samples during treatment days when receiving twice daily treatment of enteric-coated bolus containing Crofelemer. After this first design, we decided to test how this natural compound would perform under natural calf rearing conditions. The study presented in chapter four was designed to test if this compound would reduce water secretion and aid milk fed calves suffering from diarrhea under natural conditions. For this study, calves were enrolled to receive a constant dose of an extract (mixed with milk at feeding times) containing Crofelemer during the first two weeks of life. The data collected in this trial was similar to the previous study; fecal dry matter, body weight, and fluid therapy administration. Calves under crofelemer administration presented lower water content in fecal samples, fewer incidence of diarrhea, and had fewer fluid therapy interventions during the first 25 days of life. The ability of this molecule to regulate water secretion during events of secretory diarrhea is highly dependent of its ability to bind to chloride channels. Future studies should be conducted to investigate the prevalence of those chloride channels in cattle, equine, and swine.

In chapter five, we developed a study to address an important aspect of the modern replacement dairy heifer facility; the group-housing of newborn calves during

the pre-weaning period and its higher risk of calfhood disease. For this study we selected a long acting antimicrobial to test two metaphylactic approaches; one administration (at 10 days of life) and two administrations (at 10 and 35 days of life). A randomized clinical trial was conducted on a commercial dairy farm where newborn heifers were group-housed (25 calves per group) and fed unrestricted amounts of acidified milk. Diseases incidences and body weight data were collected. In this set-up we observed that both metaphylaxis treatments significantly reduced respiratory disease and otitis disease incidence. However, we could not observe an effect of metaphylaxis on calf weight gain and mortality rate. Carefully and strategically, long-acting antibiotic metaphylaxis could be used to decrease the incidence of these diseases in high-risk group-housed replacement heifers during the pre-weaning period.

In chapter six, our objective was to determine if there was an association between lung lesions detected by ultrasonography at weaning and later performance in replacement dairy heifers. This study was conducted on a commercial dairy farm where calves were classified as not having lung consolidation or with lung consolidation at 60 days of life. Productivity and reproductive performance data were retrieved from farm data records. Here, we observed a higher hazard of being removed from the herd, a lower pregnancy to first service, and a lower hazard of pregnancy for nulliparous animals with lung consolidation detected at weaning when compared to heifers without lung consolidation. However, we could not observe a difference in milk production. We proposed that thoracic ultrasonography performed once at weaning could be used as a calf-side tool to improve heifers' management.